STUDIES ON THE RUST FUNGI *UROMYCES DIANTHI* (PERS.) NIESSL AND *PUCCINIA ARENARIAE* (SCHUM.) WINT.

by

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ABSTRACT

Two rust fungi with Caryophyllaceous hosts were studied, the carnation rust Uromyces dianthi (Pers.) Niessl and the Sweet William rust Puccinia arenariae (Schum.) Wint. Their disease symptoms were investigated.

The morphology of U. dianthi urediospores and teliospores and P. arenariae teliospores and basidiospores were examined. The surface structure of germinating U. dianthi urediospores was observed by scanning electron microscope.

Experiments to determine the optimum germination temperatures of U. dianthi urediospores and P. arenariae teliospores were carried out. The optimum pH value for the germination of U. dianthi urediospores was also determined. U. dianthi urediospore exudates were investigated for possible self-germination inhibitors and various experiments performed on the inhibitory properties of the exudates.

A successful attempt was made to initate axenic cultures of U. dianthi from urediospores and the conditions necessary for the optimum growth of U. dianthi in vitro were determined. Various nitrogen and carbohydrate sources were compared as to their ability to support saprophytic colonies of U. dianthi. The phenomenon of axenic growth cessation was investigated. Attempts to culture P. arenariae from basidiospores were unsuccessful.

Experiments to observe the effects of systemic fungicides on U. dianthi cultures were undertaken.

The morphology of septal pores of axenic hyphae of U. dianthi were examined using a transmission electron microscope.

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GENERAL INTRODUCTION

The Uredinales, or rust fungi, is an interesting family of ecologically obligate parasites infecting Angiosperms, Gymnosperms and Pteridiophytes. Some rust species attack wild plants, others cultivated crops. Many ornamental plants are also susceptible to members of the Uredinales. This study is devoted to certain morphological and physiological aspects of the two rust fungi Uromyces dianthi (Pers.) Niessl and Puccinia arenariae (Schum.) Wint. both of which infect ornamental caryophyllaceous plants.

U. dianthi, the fungus causing the disease of carnation rust, has been known in Europe since 1789 when it was described by Schrank. It was said to have been introduced into Great Britain on imported carnations in 1890 (Smith,1888, 1893; Douglas,1894). Today its distribution is world wide, its presence having been reported in many countries in Africa, Asia, Europe, North and South America, and Australasia.

U. dianthi is a heteroecious rust, the urediospore, teliospore and basidiospore stages usually being found on carnations, (though they do occur on other members of the Caryophyllaceae), and the pycnospores and aeciospore stages on Euphorbia gerardiana. Infection experiments with aeciospores from E. gerardiana have been carried out by Fischer (1910, 1912), Treboux (1912) and Guyot (1938-9). Guyot and Massenot (1953) showed that E. nicaeensis was also an aecidial host. The pycnospore and aeciospore stages are unknown outside Continental Europe, and elsewhere the rust reproduces itself in the urediospore stage only.

Arthur (1907-27) described the fungus in N. America under Nigredo caryophyllinus. Grove (1913) listed the rust as U. caryophyllus, infecting Dianthus barbatus, Dianthus caryophyllus, and Dianthus chinensis. Gäumann (1959a)

listed 26 Caryophyllaceous species on which urediospores have been found and a further 15 species on which teliospores have been observed.

From Gäumann's work and other investigator's, Wilson and Henderson (1966) stated that specialised forms of U. dianthi existed on various species of Dianthus, Saponaria ocymoides and on Tunica prolifera.

The complete life cycle of the rust fungi is characterised by the successive production of five kinds of spore and is termed macrocyclic, as in the case of U. dianthi. Besides this macrocylic type of rust, there are deviating types with modified life cycles which are thought to form the main lines of evolution (Jackson, 1931).

One such deviationary line has the aecidia, uredia, and sometimes the spermogonia stages suppressed, only the teliospores and basidiospores being produced. This micro-type of life cycle does not require the alternation of hosts and the fungus is therefore autoecious. With P. arenariae, which belongs to this microcyclic group, the spermogonia, stage appears to be absent.

Where the teliospores, under the necessary environmental conditions, germinate immediately on maturity (as with P. arenariae), the micro-types are called lepto-forms. Infection in these cases can only take place by means of basidiospores.

P. arenariae has been known in this country and abroad for many years. Its presence has been reported in America, Australia (introduced), Europe and Asiatic Russia. The fungus commonly occurs on Sweet William (Dianthus barbatus) and less frequently on other members of the Caryophyllaceae. In the literature it has been described by various workers under a variety of names according to the host on which it was found.

Plowright (1884) described occurrences of Puccinia dianthi on Sweet Williams in Hertfordshire. Five years later, when reviewing British rusts and smuts, he described the fungus under the name of P. arenariae, its synonyms until then being Uredo arenariae, Puccinia moehringiae, (when found on Moehringia trinervia), Puccinia stellaria, (when found on Stellaria spp.), P. dianthi, (when found on D. barbatus), Puccinia spergulae, (when found on Spergula arvensis) and Puccinia saginae (when found on Sagina nodosa or Sagina procumbens). He considered a form found on Lychnis diurna (Silene dioica) to be a distinct species because, he claimed, it possessed uredia (Plowright, 1889).

Grove (1913) recognised two completely separate species under the names of Puccinia lychnideanum and P. saginae. The basis of his division was due to the differences in the character of the teliosori, and the apparent absence of thickening at the teliospore apex in the fungus on Sagina procumbens. This absence of thickening, he commented, may however, have been because of teliospore germination.

Wille (1915) showed from infection experiments, that spores from Melandrium rubrum (Silene dioica) would infect another species of Melandrium (Silene alba) and species of Dianthus, Sagina and Stellaria. He also showed that spores from Moehringia trinervia would infect species of Arenaria, Dianthus, Stellaria and Spergula. He concluded that a sharp specialisation between the sub-families Silenoideae and Alsinoidae did not exist.

The presence of the fungus on Herniaria ciliata, a member of the Illecebraceae, was also noted and here the name of Puccinia herniariae was given. This form was later grouped under P. arenariae by Hylander, Jørstad & Nannfeldt (1953).

Gäumann (1959b) listed 121 species that have been infected with *P. arenariae* and recognized various specialised forms based on teliospore structure.

Wilson & Henderson (1966) recognised the fungus as being of only one species, the different appearances of the sori on the various hosts being thought to depend primarily on differences of the host structure. Variations in teliospore form, size and colour were also thought to be unimportant.

Initially, the aim of the present research was to study the structure and physiology of *U. dianthi* urediospores, with special reference to germination inhibitors; and of *P. arenariae* teliospores and basidiospores. However, after the successful initiation of an axenic culture of *U. dianthi* it was considered that research with rust fungi *in vitro* would be profitable. This commended itself for four reasons.

1. The process of initiation of the axenic culture from the urediospore germ-tube to the colonising saprophytic hyphae needed to be clarified.
2. Research on the physiology of rusts was previously restricted because of the close association between the host and parasite. In axenic culture definite data on the nutrition and metabolism of *U. dianthi* would be obtained.
3. It seemed possible that new systemic fungicides could be screened for their relative *in vitro* activity against rusts, as exemplified by *U. dianthi*, by their incorporation in culture media. It was thought that this screening procedure could prove to be useful if its reliability could be substantiated.
4. The ultra-structure of rust hyphae could be investigated more easily as they would be free from host tissues.

Chapter 1 is devoted to a description of disease symptoms, and work on *P. arenariae* host range and epidemiology. The results of the initial work undertaken on *U. dianthi* and *P. arenariae* morphology and germination physiology are presented in Chapters 2 and 3 respectively. Information gained from this study proved to be useful in the *in vitro* culture initiation attempts described in Chapter 4. This chapter is an amended and expanded version of a paper published in the Transactions of the British Mycological Society (Jones, 1972).

Chapter 5 is devoted to research on finding the optimum conditions for the *in vitro* growth of *U. dianthi*. Observations on growth and experiments on the phenomenon of axenic growth arrestment are presented in Chapter 6, and in Chapter 7 work carried out with various systemic fungicides is described. Chapter 8 is devoted to electron microscope studies of hyphae growing *in vitro*.

CHAPTER 1 DISEASE SYMPTOMS, HOST RANGE AND EPIDEMIOLOGY.

1.1 INTRODUCTION

The rusts, like most fungi, possess a vegetative part, known as the mycelium, which give rise to the reproductive structures, the sori, which are formed by the massing of mycelial hyphae in certain areas of infected host tissue.

U. dianthi and P. arenariae, both non-systemic rusts, produced sori in or near the centre of infected areas. The infected areas usually became visible after an almost balanced relationship between the rust mycelium and host tissues. The incubation period, or the time taken from infection for the disease symptoms to appear was investigated. The early and mature disease symptoms, useful for diagnostic purposes, were also studied and described.

Gäumann (1959a,b) listed species that had been observed infected with U. dianthi and P. arenariae. Host range tests using some of the species recorded by Gäumann were carried out for comparison purposes. Caryophyllaceous plants not reported as hosts were also used in the experiments.

Work on the epidemiology of P. arenariae is reported. The spread of infections of U. dianthi are also discussed.

The origin and maintenance of U. dianthi and P. arenariae stocks used in all research work, are described in the materials and methods section.

1.2 MATERIALS AND METHODS.

1.21 Origin of U. dianthi used in research

Five carnations (D. caryophyllus var. Bookham Bounty) infected with U. dianthi were obtained from Mr. H. Smart, a carnation specialist, of 52, Pendesford Avenue, Tettenhall, Wolverhampton, Staffordshire, on 1st November, 1969. The

carnations were originally grown by James Douglas, Edenside, Great Bookham, Surrey. It was believed that the carnations were infected with U. dianthi before being forwarded to Wolverhampton. Urediospores from these carnations were used as inoculum to maintain disease stocks, and all urediospores used in subsequent research were derived from this initial source.

Some of the photographs of disease symptoms were taken of commercially grown glasshouse carnations obtained from Lomax Sayers Ltd., Priors Park, Elburton, Plymouth, Devon, on 14th December 1969. Pink Sim and William Sim varieties (2 years old) were found to be severely infected, but urediospores from these plants were not used as inoculum or in any other experiments.

1.22 Cultivation of host carnations used to maintain U. dianthi stocks.

U. dianthi was maintained on the carnation variety D. caryophyllus var. Grenadin Scarlet in a glasshouse. The seed of this variety, which was susceptible to the rust fungus, was obtained from Thompson and Morgan Ltd., of Ipswich. The seeds were sown in plastic trays and the young plants later transferred to plastic flower pots. The soil used throughout was a loam, peat, sand mixture (7:3:3) with 1½ oz. of lime added per bushel. The carnations were watered by hand using a plastic watering can, care being taken to ensure that water was applied only to the soil surface, and not to the foliage. This prevented urediospores on diseased carnations being washed from the sori, and as surface water is necessary for urediospore germination, it also prevented infections on healthy carnations. Diseased and healthy carnations were grown in widely separated parts of the glasshouse.

During the winter months the glasshouse was heated electrically to ensure that the temperature did not fall below 16°C. The carnations were grown under fluorescent lamps for 8 h per day.

1.23 Infection of carnations with U. dianthi.

Infection experiments were performed in a humidity cabinet. This was a large wooden framed rectangular box structure with thick polythene walls and two hinged doors. A saturated atmosphere was obtained by spraying the interior with atomized water.

Carnations to be infected were first sprayed with distilled water, and their leaves were rubbed between the fingers to remove a water repellent layer that young leaves produced on the cuticle. After rubbing, atomized water droplets no longer rolled off the leaf surface. The carnations were then placed in the humidity cabinet and sprayed with distilled water suspensions of urediospores from a small polythene spray bottle. The best method of obtaining infection was to make small amounts of very concentrated spore suspensions, and spray each plant once..

The carnations were usually left in the humidity cabinet for 48-72 h at a temperature of 18-23°C. On fine days, the temperature inside the cabinet rose rapidly due to the sun shining through the polythene sides and heating the air confined inside. This problem was overcome by masking the cabinet with newspapers.

1.24 Origin of P. arenariae used in research.

A number of Sweet Williams infected with P. arenariae were found growing in a walled garden in the grounds of Birks Hall, Cowley Bridge Road, Exeter, Devon in November 1968, and this rust was used in the initial experiments and observations.

For the axenic culture initiation experiments, infected Sweet Williams were supplied by the Agricultural Development and Advisory Service, Cambridge, in June 1971, and came from Bleak Hall Nurseries, Biggleswade, Bedfordshire.

1.25 Cultivation of host Sweet Williams used to maintain P. arenariae stocks.

P. arenariae was maintained on the Sweet William varieties D. barbatus var. Scarlet Beauty and D. barbatus var. Pink Beauty. The plants were grown in a glasshouse from seed obtained from Thompson and Morgan Ltd., of Ipswich. The seeds were sown directly into plastic flower pots containing John Innes No.6 potting compost,

1.26 Infection of Sweet Williams with P. arenariae.

Diseased Sweet Williams were placed in a humidity cabinet surrounded by young uninfected plants. The atmosphere in the cabinet was then saturated with atomized water. The aerial surfaces of the plants to be infected were covered with a fine spray of distilled water to aid infection. The plants were left in the cabinet for 48-72 h at a temperature of 18-23°C, the foliage being periodically wetted with atomised water. Air convection currents present in the cabinet were thought to have aided the dispersal of the basidiospores.

1.27 Infection of Caryophyllaceous plants in host range experiments.

Twenty-two different species of Caryophyllaceous plants were selected from Thompson and Morgan (Ipswich) Ltd., and French's (Exeter and Sidmouth) Ltd., seed catalogues. The plants chosen represented 14 genera of the Caryophyllaceae. All were sown directly into pots containing John Innes No.6 potting compost.

When the young plants reached a stage in their development

where it was considered that they possessed a sufficient amount of foliage for the innoculation experiments, they were placed in humidity cabinets. Two plants of each species were either sprayed with suspensions of U. dianthi urediospores (as in 1.23) or surrounded by Sweet Williams infected with P. arenariae (as in 1.26). Where U. dianthi was being used, some uninfected carnation plants were also placed in the humidity cabinet at random to act as control plants. When their symptoms became apparent they gave an indication of inoculum potential to which the other plants were exposed. Uninfected Sweet Williams were placed in the cabinets in infection experiments, involving P. arenariae. After 48 h in high humidity and surface moisture conditions, the plants were removed from the humidity cabinets.

1.28 Methods used for studying the epidemiology of P. arenariae.

A 7 ft. end section of a row of D. barbatus was used in an experiment to observe the development and spread of P. arenariae. The row was growing in a sheltered walled garden behind St. Thomas Hall, Cowley Bridge Road, Exeter, Devon. The Sweet Williams had been sown in June 1968, and the young plants had been exposed to infection from a nearby badly diseased Dianthus bed in August and September of the same year. The result of this exposure was a number of late lesions, some of which survived the winter.

The row, which ran in a north-south direction, was divided into ten roughly equal areas. Each area was marked out, and consisted usually of a main Sweet William plant with subsidiary side growths. Area 1 was the first plant in the row. The areas were observed carefully every week for six weeks in May and June 1969, and the number of P. arenariae lesions noted. The progress of the epidemic was related to weather reports

obtained from the Department of Geography, The University of Exeter.

1.3 RESULTS

1.31 Disease symptoms on carnations caused by U. dianthi.

Between 15 and 24 days after infection, small pallid spots appeared on the leaf surface. These were approximately 2 mm in diameter and were equally distinct on both upper and lower surfaces. About 3 days after their appearance, urediosori could be seen developing. This was indicated by a bulging and whitening of the leaf surface near the centre of the chlorotic area. A few days later the grey-white epidermis was ruptured and mature urediospores exposed. The sporulation period (i.e. time from infection to spore production) varied from 20 to 30 days.

The size and spread of the infected zone seemed to depend on the resistance of the tissues to hyphal invasion. This was related to the carnation variety, its age, and the location of the infected area.

On young leaves, lesions tended to elongate along the plane axis of the leaf (Pl. 1.1). This indicated that less resistance was encountered by the colonizing hyphae growing along the leaf blade than those growing across it. Infections could either produce a large urediosorus with associated small scattered ones on the periphery of the growing area (Pl. 1.1) or many scattered individual sori. This latter type however was more often associated with multiple infections (Pl. 1.2). Urediosori were produced in roughly equal numbers on both the upper and lower leaf surfaces. Withering and chronic chlorosis occurred on badly infected leaves. Occasionally islands of green tissue could be seen surrounding urediosori (Pl. 1.3). This was in contrast to the yellowing generally associated

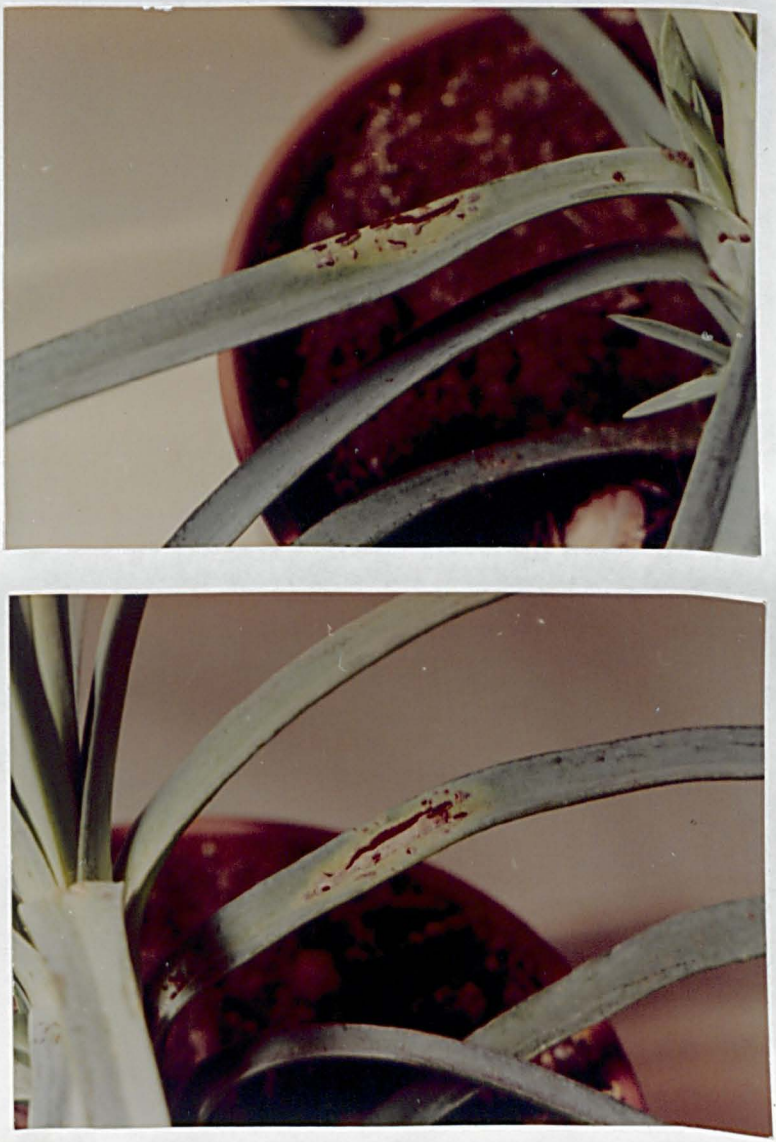


Plate 1.1. *U. dianthi* urediosori on *Dianthus caryophyllus* var. Bookham Bounty leaves.

Plate 1.2. Small scattered U. dianthi urediosori on Dianthus caryophyllus var. Bookham Bounty leaves.

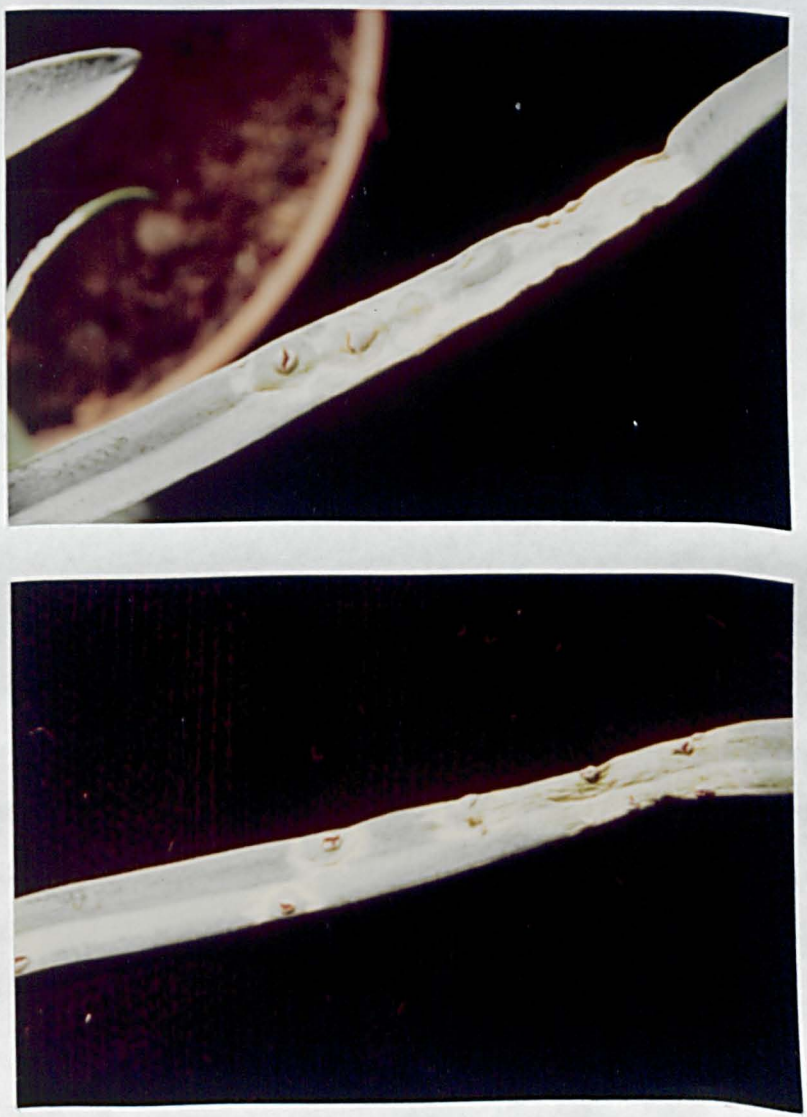


Plate 1.3. Green islands around U. dianthi urediosori on Dianthus caryophyllus var. Grenadin Scarlet leaves.

with lesions. These green islands persisted for some time surrounded by senescent leaf tissue.

Carnation stems could also become very badly infected (Pl. 1.4).

Moderately and severely infected plants failed to produce flowers.

1.32 Disease symptoms caused by P. arenariae on Sweet Williams.

The first indication of infections by P. arenariae was the appearance of small circular chlorotic spots on the leaves. There were visible on the upper leaf surface, but were much more obvious and more sharply defined on the lower. The incubation time was 12 days.

When they first became noticeable, the spots were about 2-3 mm in diameter. When they reached a diameter of 3-4 mm, reddish tinges could be seen under the leaf surface in the centre of the spot. These were caused by the developing teliospores under the epidermis. The teliospores were observed in some instances to appear almost simultaneously with the chlorotic spot symptoms. The sporulation period was 15 days.

Mature symptoms seemed to depend on the intensity of infection and the age, composition, and resistance of the tissues. With single localised infections, circular chlorotic spots were formed which, when fully developed, were about 6-7 mm in diameter. The largest spot seen was 11 mm in diameter. On the lower surface brown-black teliospore masses were seen protruding through the ruptured epidermis (Pl. 1.5). Sometimes almost circular patterns of teliosori could be seen, formed in concentric zones around the initial point of entry. Occasionally, teliospore masses broke through the upper epidermis (Pl. 1.6).

The circular spot was the distinctive symptom, but

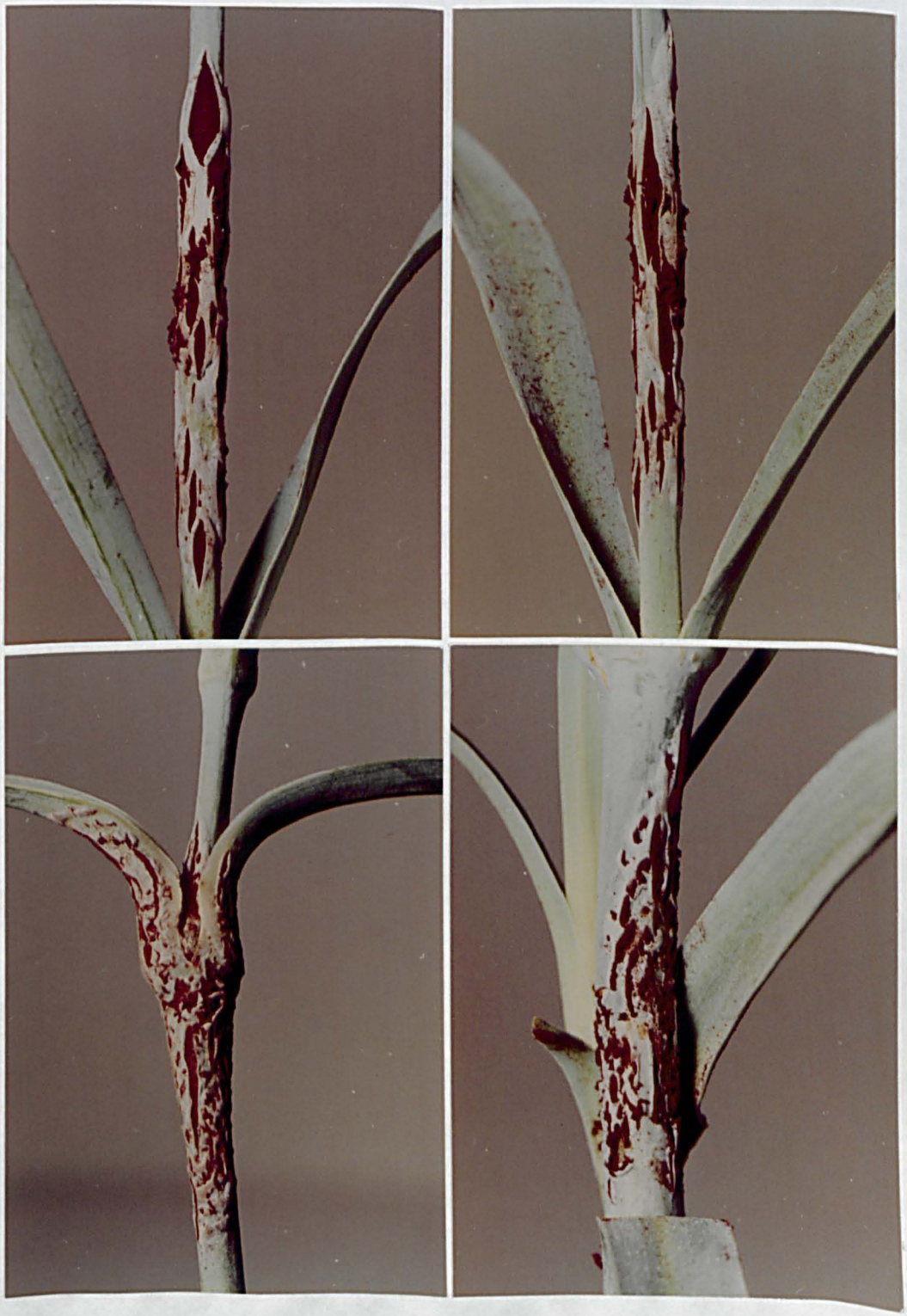


Plate 1.4. U. dianthi urediosori on Dianthus caryophyllus var. Pink Sim stems.

Plate 1.5. P. arenariae teliospori on the lower surface of a Dianthus barbatus var. Scarlet Beauty leaf.

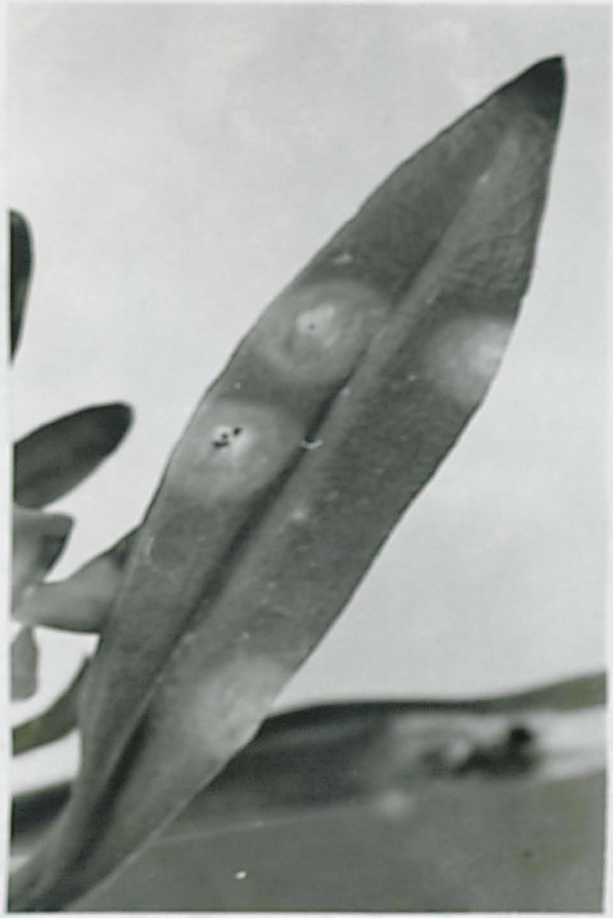


Plate 1.6. The upper surface of a Dianthus barbatus var. Scarlet Beauty leaf showing large circular chlorotic spot symptoms of P. arenariae.

Plate 1.7. Elongated lesions and elliptical teliospori patterns on a Dianthus barbatus var. Scarlet Beauty stem infected with P. arenariae.

elongated lesions were also found, especially near the petiole of a leaf. The oval shape of these lesions could be accounted for by faster growth of the pathogen in directions parallel to the vein due to less mechanical resistance. Elongated lesions were also occasionally found on the stems (Pl. 1.7). This seemed a rare occurrence however, stem infections being observed only twice in the course of the investigations. Occasionally teliosori were observed on the sepals of flowering plants.

In cases where infections were severe, the circular and oval lesions coalesced to form a large yellowed area containing many scattered teliosori (Pl. 1.8). This often resulted in a totally chlorotic distorted leaf. The multiple rupturing of the epidermis by the teliosori led to the evaporation of moisture and the eventually withering of the leaf. The ruptured leaf surface also enabled secondary pathogens to gain entrance to the tissues. Intense infections also favoured the production of teliosori on the upper leaf surface. This, as mentioned previously, was only rarely seen with isolated or moderate infections.

When the teliospore mass germinated, the promycelia produced gave the teliosori a greyish color.

After spore production, the centre of the old lesion dried, withered and dropped out, leaving a hole in the leaf tissue. There was also evidence suggesting that some insects hastened this process by preferential feeding on the chlorotic areas.

On one occasion a small unnatural growth was observed to arise from a moderately infected area, but generally the pathogen did not seem to cause any noticeable hypertrophy. Guyot (1931) however, described the occurence in France of

Plate 1.8. The lower surface of a Dianthus barbatus var. Scarlet Beauty leaf severely infected with P. arenariae

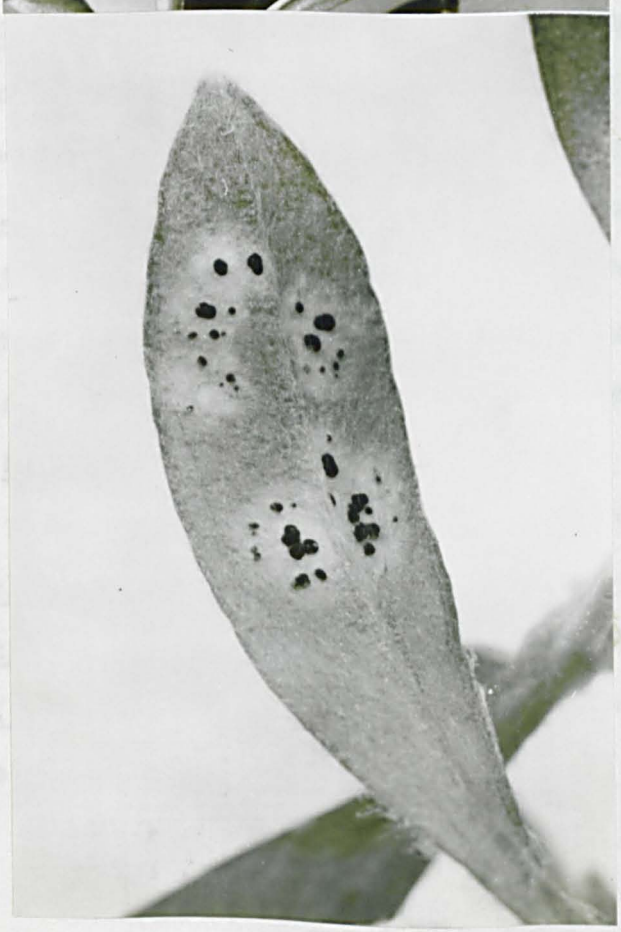


Plate 1.9. The lower surface of a Cerastium tomentosum leaf showing P. arenariae teliosori.

witches broom effect on Moehringia trinervia due to infection by P. arenariae.

1.33 Host range experiments.

The species selected for the host range experiments were Agrostemma milas, Arenaria montana, Cerastium tomentosum, Dianthus arenarius, D. barbatus, D. caryophyllus, Dianthus plumaris, Dianthus deltoides, Gypsophila elegans, Gypsophila paniculata, Hernaria glabra, Lychnis chalcedonica, Lychnis coronaria atrosanguinea, Melandrium rubrum, Petrocoptis legascae, Sagina sublata, Saponaria calabrica, Saponaria ocymoides, Silene ocaulis, Silene pendula compacta, Tunica saxifraga, and Viscaria candida.

The control plants used were moderately to severely infected, indicating that all plants were exposed to a reasonably high inoculum potential.

U. dianthi infected and produced spores on D. caryophyllus (control) and D. barbatus. Early symptoms, but no spores were observed on G. elegans. Small white necrotic spots on A. milas and D. plumaris indicated that infection had occured but that the tissue of these plants produced a hypersensitive reaction. No other symptoms of infection were found on any of the other plants. Infection appeared not to take place.

P. arenariae produced teliospores on A. milas, C. tomentosum (Pl. 1.9), D. arenarius, D. barbatus (control), D. caryophyllus, D. deltoides, D. purpureum, G. elegans, P. legascae, S. sublata, S. acaulis and T. saxifraga. White necrotic spots were observed on A. montana and S. ocymoides. No other species showed any signs of infection.

1.34 Epidemiology of P. arenariae.

The observations on 9th May showed the number of overwintering lesions (Table 1.1). These lesions had, in fact,

been observed on the plants during the winter months, and all were found on old leaves, possessed mature teliosori, and were all sources of inoculum.

TABLE 1.1 Weekly observations on a row of Dianthus barbatus plants infected with P. arenariae

Date of Readings	No. of Lesions per Area									
	Area 1	Area 2	Area 3	Area 4	Area 5	Area 6	Area 7	Area 8	Area 9	Area 10
May 9th	0	0	0	4	2	1	0	0	0	0
May 16th	0	0	21	187	36	35	6	11	11	19
May 23rd	0	3	33	339	39	36	24	18	19	27
May 30th	0	2	29	448	27	43	11	7	10	20
June 6th	0	2	34	298	29	19	3	0	2	11
June 13th	222	319	997	>1000	821	580	615	393	350	422

The May 16th totals represented in the main new lesions on new growth. They appeared fairly evenly distributed over the infected plants, with occasional concentrations on some leaves. This dramatic increase in one week could be explained by a period of exceptionally wet weather during the first few days of May, about 14 days previously (incubation period 12 days). Average temperatures and humidities recorded by the Geography Department in the grounds of Exeter University at this time were around 10°C and 80% r.h. These readings were taken in an exposed position, but the gardens at Thomas Hall were in a much more sheltered locality. The humidity among the plant foliage was thought to be much higher. In early May, during this wet period, the overwintering lesions were, in fact, observed to have grey teliosori due to teliospore germination and promycelial growth indicating 100% r.h.

The areas with the largest number of surviving overwintered lesions showed the largest initial build-up of new lesions. Spread outwards also occurred to some extent, and previously clean areas became infected. The apparent escape of areas 1 and 2 indicated that in this instant, the basidiospores did not travel very far. It could have been however, that the prevailing air currents in the garden at that time were north-south, down the row, as indicated by infections in areas 7, 8, 9 and 10.

The May 23rd totals showed another significant increase in the number of lesions in the badly infected area 4. This may have been due to the early May infections developing symptoms after the readings taken on May 16th. Slight increases in other areas were also recorded and three lesions appeared in previously clean area 2.

Observations on May 30th revealed an overall drop in

the number of lesions in most areas. The reason for the decline appeared to be decay, and falling-out of many lesions. Here area 4 was the only one to show an increase.

The June 6th totals showed another overall reduction in the number of lesions, with an appreciable fall in area 4. The probable explanation for this was the comparatively dry weather which occurred in mid-May. Conditions then did not favour teliospore germination, and older lesions continued to decay.

A prolonged warm wet spell (average temperature 17°C, average humidity 90% r.h. at University) at the end of May seemed to be responsible for the considerable increases in lesions in the June 16th totals. All areas became badly infected, and must have been subjected to a very heavy inoculum potential.

From the above results, and other evidence, a disease development pattern was derived.

Early May - Suitable environmental conditions (i.e. mild wet weather) leads to the development of a number of secondary infection centres from the few overwintering primary foci.

Summer months - Subsequent build-up of infection. This takes place in stages, massive increases occurring during suitable environmental periods. Between these periods, there can be reversals in the numbers of lesions due to decay of the diseased tissues.

Late Summer, early Autumn - Inoculum from old diseased Sweet Williams plants infects newly sown, young, developing plants in the same area. Sometimes infection can be so severe that they are destroyed. At other times the inoculum potential may be lower and only a small number of lesions may develop, which subsequently overwinter.

As mentioned above, P. arenariae can severely attack some

young varieties of D. barbatus. Epiphytotics seem to occur only in the south (climatic conditions), and one was reported to have occurred at Thomas Hall, Exeter, during the wet summer of 1968. Here a whole bed of a rather susceptible hybrid variety was completely destroyed. The attack was apparently so damaging that by September, the plants were devoid of any green foliage and the remains were dug into the soil.

1.4 DISCUSSION.

Lesion size, as indicated by chlorotic areas, seemed to be generally larger in U. dianthi infected carnations than in P. arenariae infected Sweet Williams. The reason for the limitations in lesion size is not fully understood, but it is thought to be the result of a dynamic defence reaction stimulated in the host tissues by the invading hyphae.

The reason for the change in the host-pathogen relationship which brings about symptom expression is also not known. The presence of the rust mycelium obviously has a deleterious effect on the host tissue and the change could be due to the build up to a critical level of metabolites toxic to the plant cell. The overall effect is the gradual destruction of the cell chloroplasts. The green islands which appear around rust pustules on some chlorotic leaves however, indicates the re-stimulation of chlorophyll production. This has been related to localised increases in growth regulating substances (Wood, 1967). Initial symptom expression on the host seems related to rust spore production.

The sporulation period or generation time is an important factor when one considers the epidemiology of a fungus. U. dianthi was found to vary between 20 and 30 days, whereas P. arenariae was 15 days.

In Britain U. dianthi is fairly common on glass house carnations, but is rarely found on garden carnations. This is probably due to climatic factors. In large commercial glasshouses in the South West of England epiphytotics have been known to occur. Rows of severely diseased carnations were noticed to be situated in central areas of the glass-house where moisture gathered on the glass panes and dripped on the foliage below. The spread of U. dianthi is therefore almost certainly due to a combination of urediospore dispersal in water drops running from diseased to neighbouring healthy plants, or to human transferance of spores when cutting flowers etc.

From work on its epidemiology, P. arenariae was found to be a pathogen which can quickly multiply under favourable conditions, spreading rapidly amongst neighbouring plants.

Gäumann (1959a) found U. dianthi on D. arenarius, D. deltoides, D. plumaris, S. ocymoides and T. saxifraga which were not infected in the host range experiments.

Similarly, P. arenariae failed to infect A. montana, G. paniculatum and M. rubrum. Gäumann (1959b) however lists these plants as hosts. New host species recorded for P. arenariae were A. milas, D. arenarius, P. legascae and S. acaulis. It was thought that different races of U. dianthi and P. arenariae may infect a different range of hosts. If proven, host range experiments could be used to identify races of these rusts.

CHAPTER 2 SPORE MORPHOLOGY.

2.1 INTRODUCTION

In this chapter the structure of urediospores and teliospores of *U. dianthi* and the teliospores, promycelia, and basidiospores of *P. arenariae* are described. Germ-tubes and infection structures of *U. dianthi* urediospores and *P. arenariae* basidiospores were also investigated.

Grove (1913) described *U. caryophyllinus* (*U. dianthi*) urediospores as globose to ellipsoid, sparesly echinulate, yellowish-brown, 20-35 x 18-25 µm; wall 2.5-3 µm thick, with 3-5 germ-pores. Wilson and Henderson's (1966) description agreed with that of Grove (1913). In the C.M.I. descriptions of pathogenic fungi and bacteria No.180 (Punithalingam, 1968) *U. dianthi* urediospores were noted to be broadly ellipsoid, 20-24 x 24-30 µm, wall golden brown, 2.5-3 µm thick, strongly echinulate, pores 3-4, equatorial.

The ornamentations on urediospores has been considered by some workers to have possible diagnostic significance (Corlett, 1970). It has been suggested that not only could species of rusts be identified by the dimensions of their ornamentations, but also races of rusts. By the use of carbon replicas, Payak, Joshi & Mehta (1967) found differences in the halo-like areas surrounding surface spines in races 24 and 42 of *P. graminis* f. sp. *tritici*. Stanbridge & Gay (1969) however concluded that no significant differences existed between the urediospore ornamentation dimensions of four races of *Puccinia striiformis*. The ornamentation features of *U. dianthi* urediospores were investigated.

Prominent features observed in light microscope studies of germinated rust urediospores have been the differential areas in the spore walls through which the germ-tubes emerged.

The arrangement and structure of these germ-pores was considered by Arthur & Fromme (1915) to have taxonomic importance, and by Cummins (1936) to have phylogenetic significance. The arrangement, number and structure of germ-pores in U. dianthi urediospores was reinvestigated in the present work. A scanning electron microscope study was also undertaken to observe if any external morphological features indicated the location of the germ-pore before germination, and the effect of germination on the spore wall in the germ-pore region. A report on the observations made using the scanning electron microscope appeared as a note in the Canadian Journal of Botany (Jones, 1971).

The form of the infection structures of U. dianthi on the surfaces of carnation and Sweet William leaves were observed and compared.

The teliospores of U. dianthi were described by Grove (1913) as globose to ellipsoid, with a flat hyaline papilla, densely and minutely punctate, chestnut brown, 20-31 x 18-24 µm; wall 2-3 µm thick, not thickened at the summit; pedicels short, hyaline deciduous. The description by Wilson & Henderson (1966) agreed with Grove's, but adds that the flat hyaline papilla is apical and that the spore is densely and minutely echinulate. In the C.M.I. descriptions of pathogenic fungi and bacteria No.180 (Punithalingam, 1968), the teliospores are said to be 'ellipsoid, 20-23 x 25-29 µm, rounded above and below, wall light chestnut brown, 3 µm thick with hyaline papillae over the germ pore, finely verrucose, pedicel colourless, short. U. dianthi teliospores were examined and described.

Plowright (1889) described the teliospores of P. arenariae as being 'broadly fusiform or pyriform, summits pointed or

rounded, often thickened, base rounded or attenuated, slightly constricted, smooth, pale yellowish brown 30-50 x 10-20 µm. Pedicels hyaline, colourless, as long as the spores.

Grove (1913) notes the teliospores as being oblong-fusoid or clavate, rounded or somewhat pointed above and more or less thickened (up to 10 µm), gently constricted, rounded or attenuated below, smooth, yellowish-brown, 30-50 x 10-20 µm, pedicels hyaline, persistent, 60-85 µm long. The description of Wilson & Henderson (1966) was very similar to that of Grove (1913).

The structure of P. arenariae teliospores was investigated on a number of host species. The development of the basidia or promycelia from germinating teliospores was also described. In the literature, there seemed to be two contrasting opinions on the anatomical structure of the basidium. Illustrations by Grove (1913) of P. lychnidearum (P. arenariae) showed 4-celled basidia, each cell apparently capable of producing a basidiospore. Lindfors (1924), on the other hand, stated that a 2-celled basidium developed from germinating teliospores, each cell producing one basidiospore.

The form and nuclear migration in germinating basidiospores were investigated, and also the formation of infection structures on the leaf surface.

2.2 MATERIALS AND METHODS

2.21 Origin and collection of spores.

U. dianthi urediospores and teliospores were collected from infected carnations grown in the glasshouse (see Chapter 1.21, 1.22). The sori were brushed with a camel hair paint brush and the spores collected in a plastic Petri dish.

P. arenariae teliospores were obtained by the fragmentation

of teliosori cut from infected plants grown in glass-houses at Exeter (see Chapter 1.24, 1.25). Basidiospores developed on basidia formed on the germination of teliospores.

2.22 Spore mounting and staining techniques.

U. dianthi urediospores and teliospores were unstained and mounted in lactophenol. For photographic work, the spores were mounted in water. P. arenariae teliospores were stained and mounted in cotton blue and lactophenol. Basidiospores were discharged over glass cover slips. Small staining blocks were used as humidity chambers, teliosori being fastened to the inside of the glass plate lid with adhesive tape. The basidiospores were either mounted in lactophenol and cotton blue, or immersed in Singleton's fixative and stained with Giemsa. All the staining was performed with the basidiospores adhering to the cover slips.

Drawings were made using a camera lucida. Measurements were taken with a calibrated eye piece.

2.23 Leaf surface studies.

Young detached carnation and Sweet William leaves were sprayed with atomised water and floated on the surface of distilled water in plastic Petri dishes. U. dianthi urediospores were then lightly dusted over the leaf surfaces. The Petri dishes were incubated at 18°C for 12 h. The leaves were decolourised in saturated chloral hydrate solution. The spores, germ-tubes, and infection structures were then stained by immersion in a dilute solution of trypan blue in lactophenol. After 15 min, the stain was drained off, and pieces of the leaf were mounted in glycerol.

Young leaves of D. barbatus were floated on distilled water in large staining blocks. The leaf surfaces were then covered with a fine spray of distilled water. Teliosori were then attached with adhesive tape to the inside of the staining

block glass covers and placed in a position over the leaves. The covers were sealed on the block with petroleum jelly. Time was allowed for germination of the teliospores, discharge of basidiospores and their germination on the leaf surface. The leaves were then cleared and the basidiospores stained in the same way as U. dianthi urediospores.

A camera lucida was used to enable accurate drawings to be made.

2.24 Scanning electron microscope studies.

U. dianthi urediospores were dusted over a thin layer of 1.5% water agar in a Petri dish. The spores were incubated at 18°C for 12 h, and then fixed by spraying the agar surface with atomized 3% glutaralderhyde solution. The agar was then cut into small pieces, attached to specimen holders, and coated with a thin film of gold-palladium alloy under high vacuum. Observations were made with a Cambridge Stereoscan Electron Microscope (Mark II-A) at the Department of Botany, University of Liverpool, England (Operator Mr. C.J. Veldkamp).

2.3 RESULTS

2.31 U. dianthi urediospores.

2.31.1 Light microscope observations.

The outside spore wall was found to be covered with numerous minute projections (Fig. 2.1). The inside wall was smooth, but in some spores it was undulating. The thickness of the spore wall varied, being thicker in some parts than others. In cross-section, the germ-pore regions appeared as areas where the spore wall was less opaque, indicating a less dense matrix (Pl. 2.1). Examination of empty germinated urediospores showed that each spore possessed between 2 and 5 circular equatorial distributed germ-pore regions (Fig. 2.2).

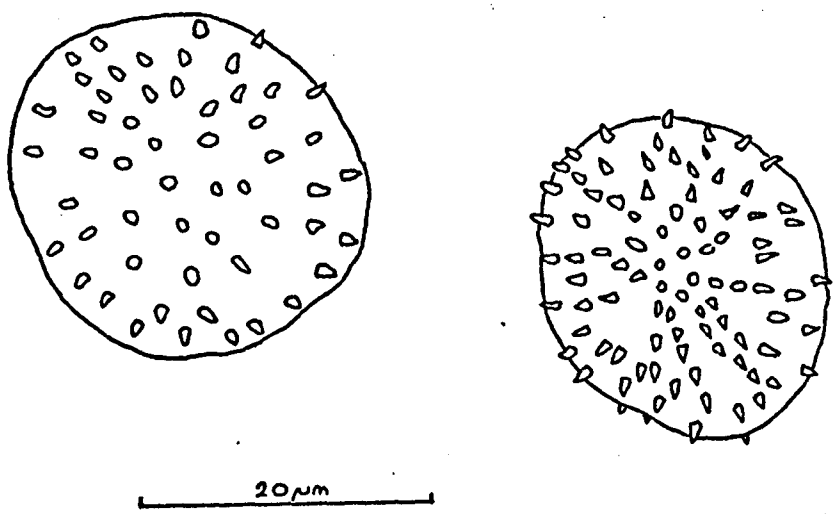


Fig. 2.1. U. dianthi urediospores showing surface projections.

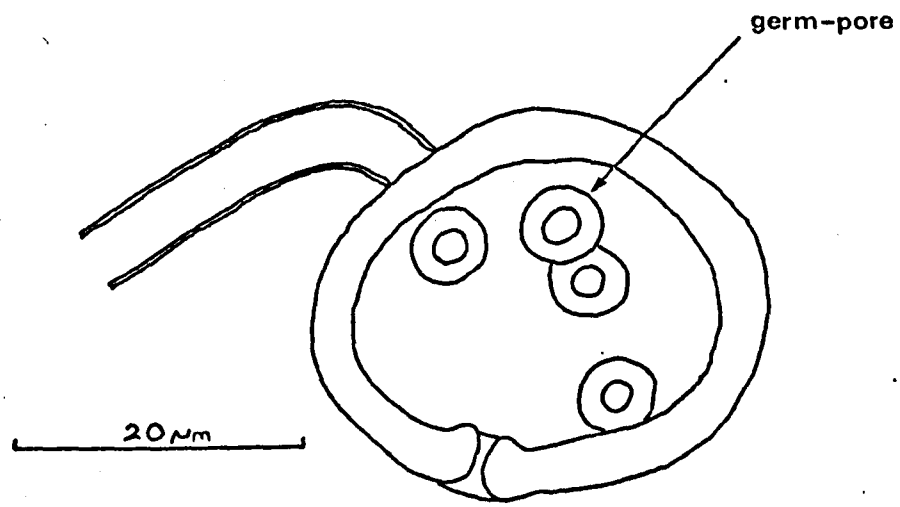


Fig. 2.2. An empty U. dianthi urediospore showing five germ-pores.

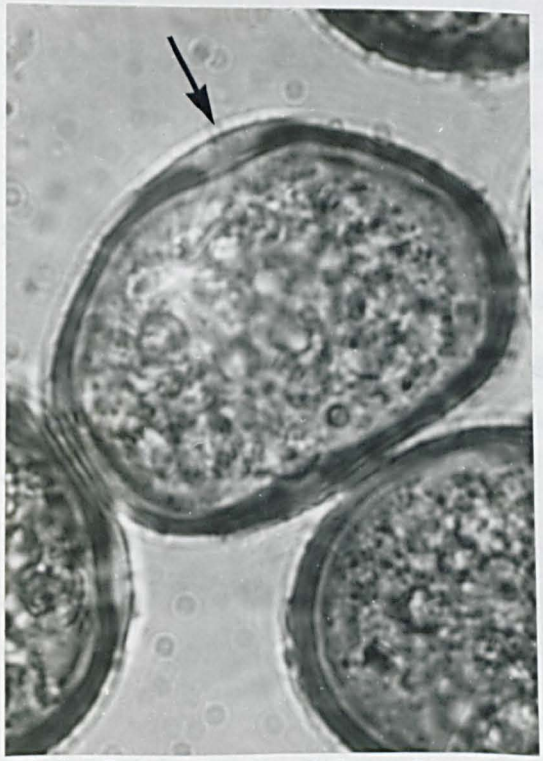


Plate 2.1. U. dianthi urediospore showing germ-pore region (arrow). X 2640.

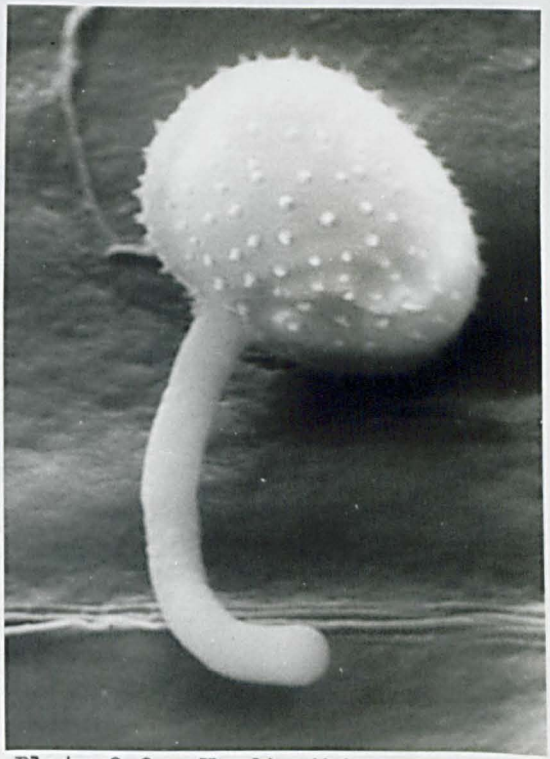


Plate 2.2. U. dianthi urediospore with germ-tube. X 2150.

Plate 2.3. Area around an emerged U. dianthi urediospore germ-tube. X 5830.

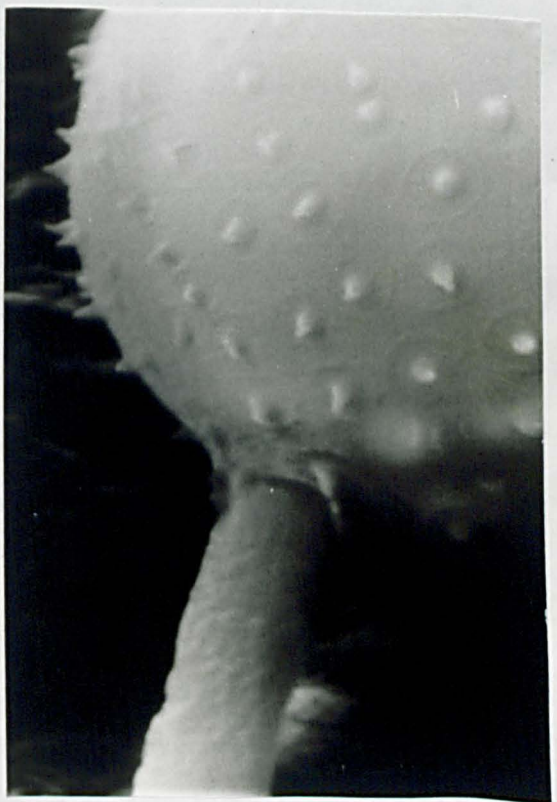


Plate 2.4. As in Plate 2.3. X 5400.

Usually the urediospore contained one or two translucent bodies, believed to be large aggregations of lipid vesicles.

From many observations the urediospores (Fig. 2.3) were described as:-

Globose to ellipsoid, 22-32 x 20-26 μm; wall yellowish-brown, 2.3-3.5μm thick, moderately echinulate; pores 2-5, 2-2.5 μm in diameter, equatorial.

2.31.2 Scanning electron microcscope studies

About 50 spores in various stages of germination were examined. Inhibition effects in areas of high urediospore density (see Chapter 3.33) resulted in some spores showing no signs of germination. No obvious external morphological features indicated the position of germ-pore regions on these spores. Slight depressions in the surfaces of some spores were thought to be areas where weak germ-pore regions had partially collapsed in the vacuum.

Some urediospores possessed germ-tubes that had not collapsed in the vacuum (Pl. 2.2). These were examined and they showed no splitting or distortion of the spore wall adjacent to the emerged germ-tubes (Pl. 2.3 & 2.4). There also appeared to be no swelling of the germ-tube after emergence.

The surface of the urediospores was covered with small, curved, conical spines, 0.7-1.0 μm in height (Pl. 2.5a). These were usually set singly in very shallow depressions ringed by circular ridges 1.0-2.0 μm in diameter (Pl. 2.5b). In some places two spines were surrounded by a common ridge (Pl. 2.6).

2.31.3 Germ-tubes and infection structures.

The diameter of germ-tubes varied between 4.5 and 7.0 μm. On the carnation leaf surface germ-tubes were seen to extend from urediospores to stomata. Appressoria were seen over stomata. Sub-stomatal vesicles with developing infection

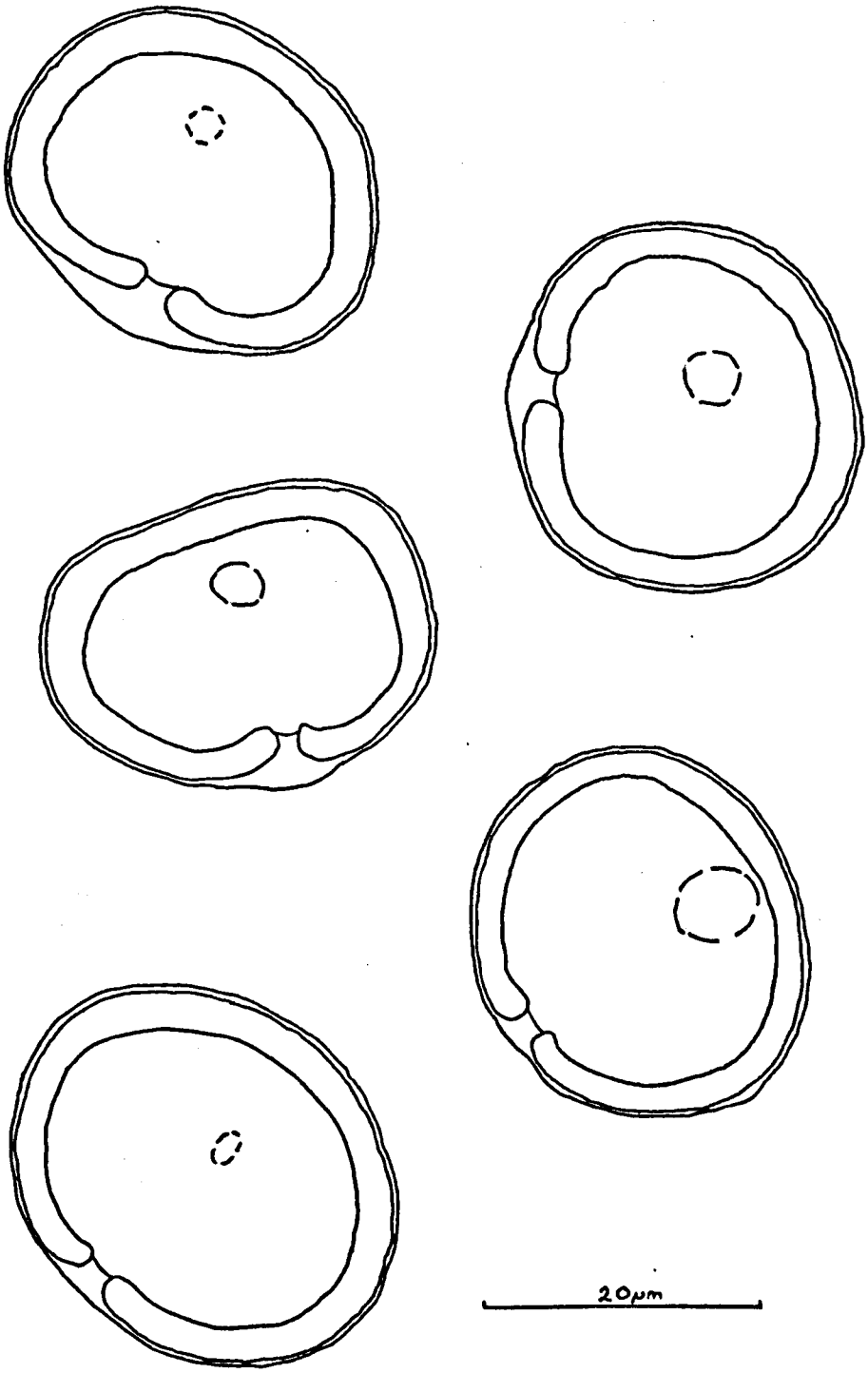


Fig. 2.3. U. dianthi urediospores.
Bodies, believed to be aggregations of lipid vesicles, are visible in the spores.

Plate 2.5a. Spine-like ornamentations on a U. dianthi urediospore. X 55650.

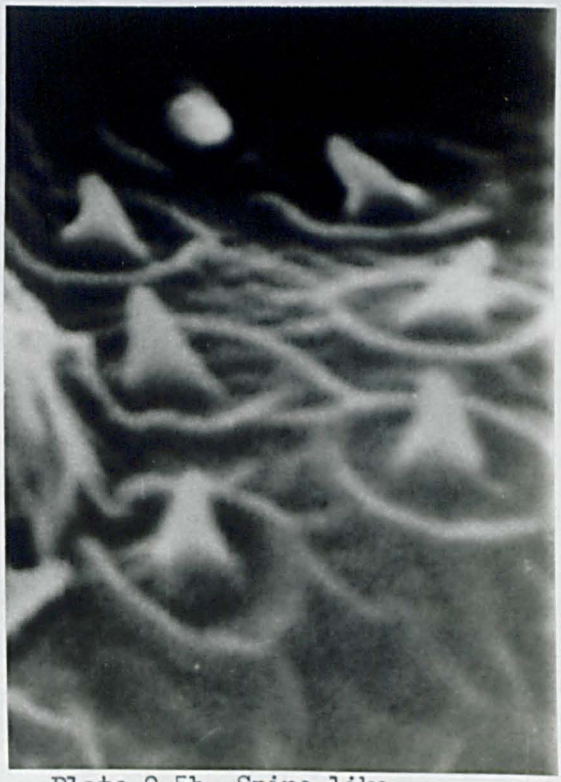


Plate 2.5b. Spine-like ornamentations surrounded by circular ridges on a U. dianthi urediospore. X 18060.

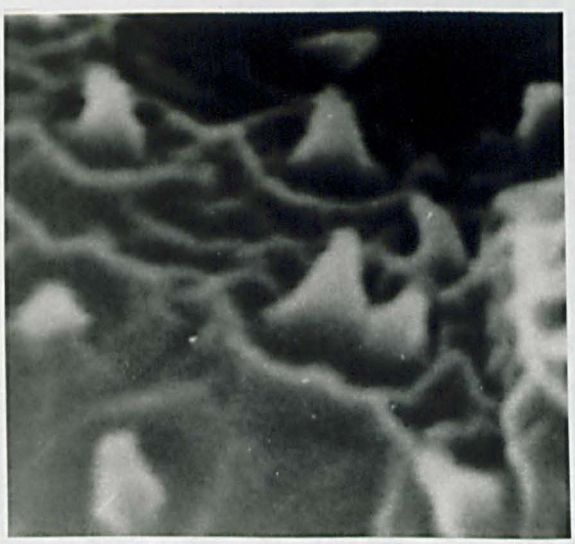


Plate 2.6. Double spine features on a U. dianthi urediospore. X 18060.

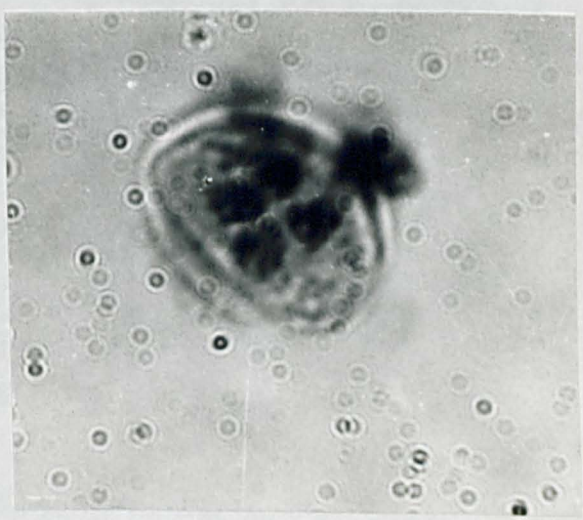


Plate 2.7. P. arenariae basidiospore showing four nuclei. X 2330.

hyphae were observed (Fig. 2.4). Usually the germ-tube cytoplasm flowed to the appressorium. If the urediospore was adjacent to a stoma, the appressorium was formed on germination (Fig. 2.5). Usually one appressorium was formed per stoma. In areas of high urediospore concentration, germ-tubes were observed growing past occupied stomata. One germ-tube was measured and found to be 160 µm long before appressorial formation. Occasionally however, two appressoria were found occupying one stomatal site (Fig. 2.6). On D. barbatus var. Scarlet Beauty leaves the majority of germ-tubes failed to differentiate appressoria, even though some crossed stomata. Some germ-tubes did form infection structures however, though these were usually not over stomata (Fig. 2.7). Unlike the germ-tubes on carnation leaves, there seemed to be no directional growth towards stomata.

2.32 U. dianthi teliospores.

Teliospores were observed by the light microscope. They appeared to have smooth external and internal spore wall surfaces. The spore wall varied in thickness, usually being thickest at the apex because of a raised area over the germ-pore. Each spore possessed one germ-pore, and this was almost always located at the spore apex. This area was less dense than the surrounding spore wall. Hyaline stalk cells were usually attached to teliospores. These could be up to 88 µm in length. A channel of less dense matrix in the teliospore wall linked it with the pedicel. Spherical translucent bodies in the cytoplasm of some spores were thought to be lipid vesicles.

From observations on a number of teliospores (Fig. 2.8), they were described as:-

Globose to ellipsoid, 23-24 x 17-23 µm, wall light chestbut (amber) brown, 2-3.5 µm thick, with a hyaline papilla

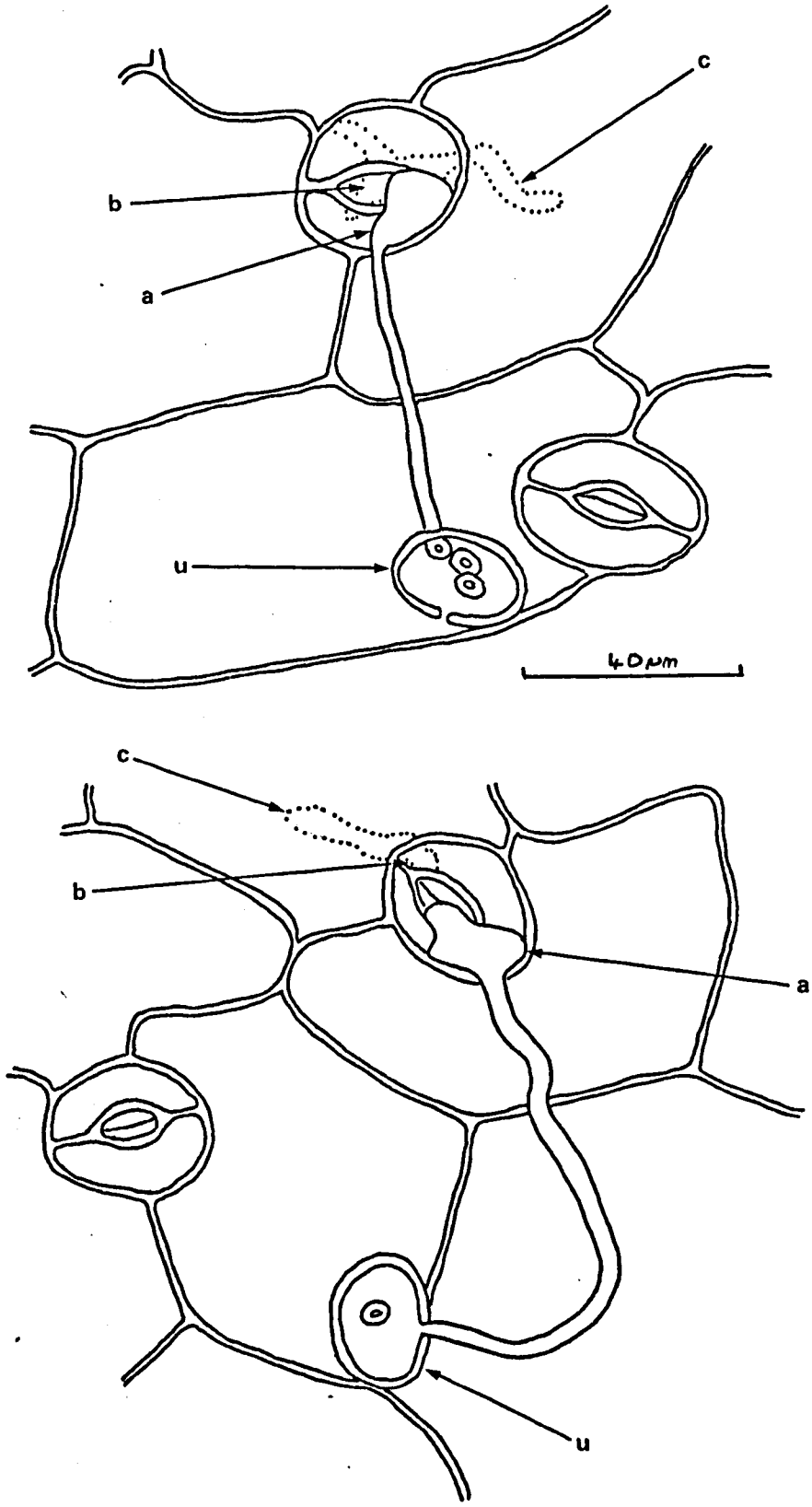


Fig. 2.4. Germinated U. dianthi urediospores (u) on a carnation leaf surface showing the differentiation of the germ-tube to appressoria (a), sub-stomatal vesicles (b) and developing infection hyphae (c).

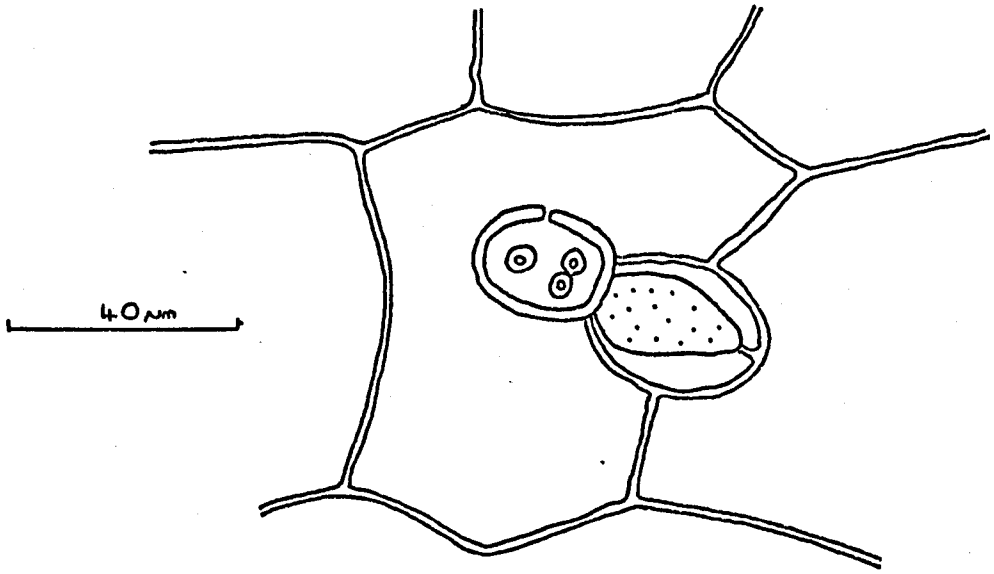


Fig. 2.5. A germinated U. dianthi urediospore forming an appressorium over a carnation leaf stoma.

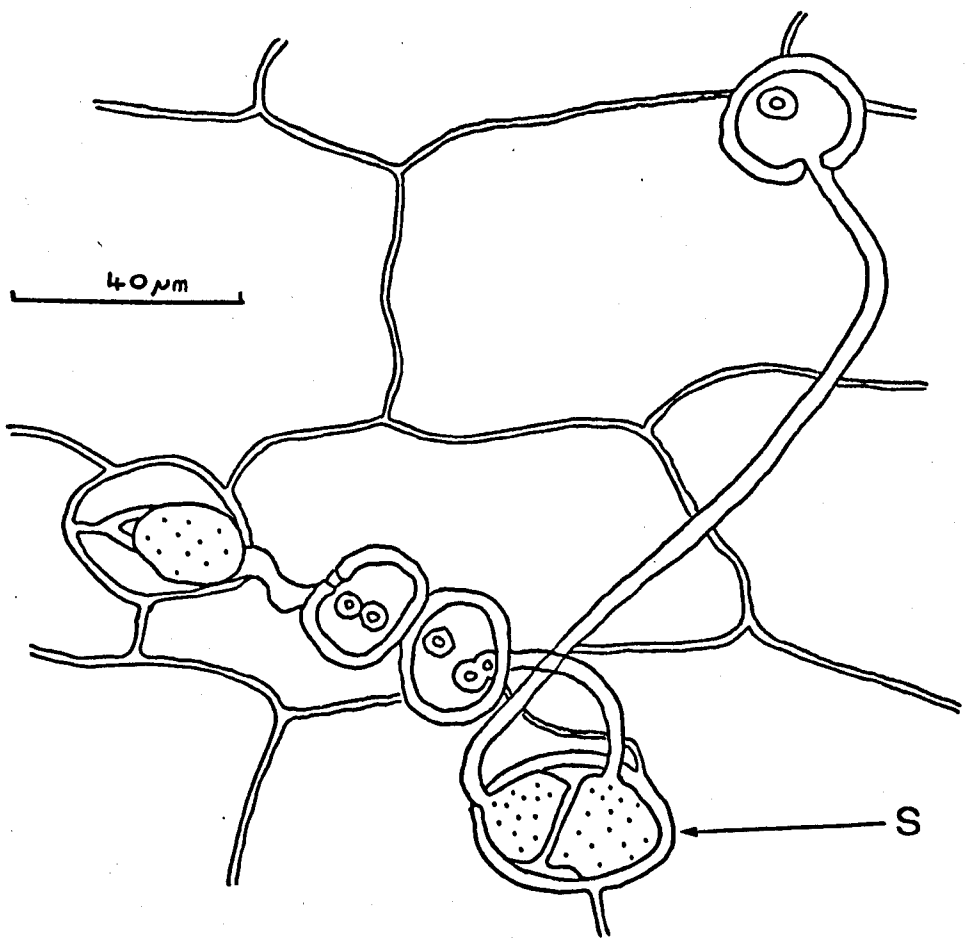


Fig. 2.6. Two differentiated appressoria from U. dianthi urediospore germ-tubes occupying one carnation leaf stomatal site (s).

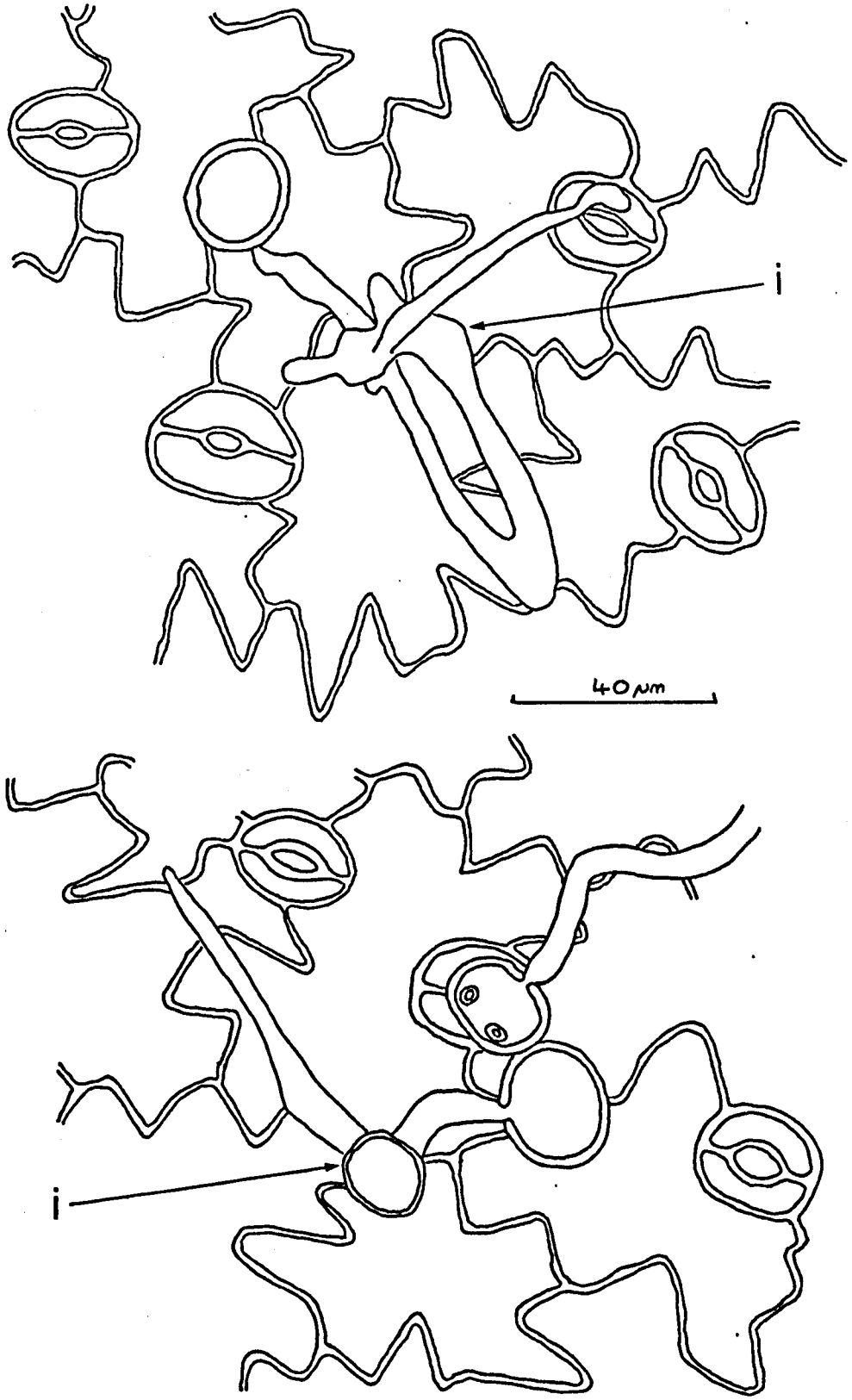


Fig. 2.7. Infection structures (i) formed by U. dianthi urediospore germ-tubes on Sweet William leaves.

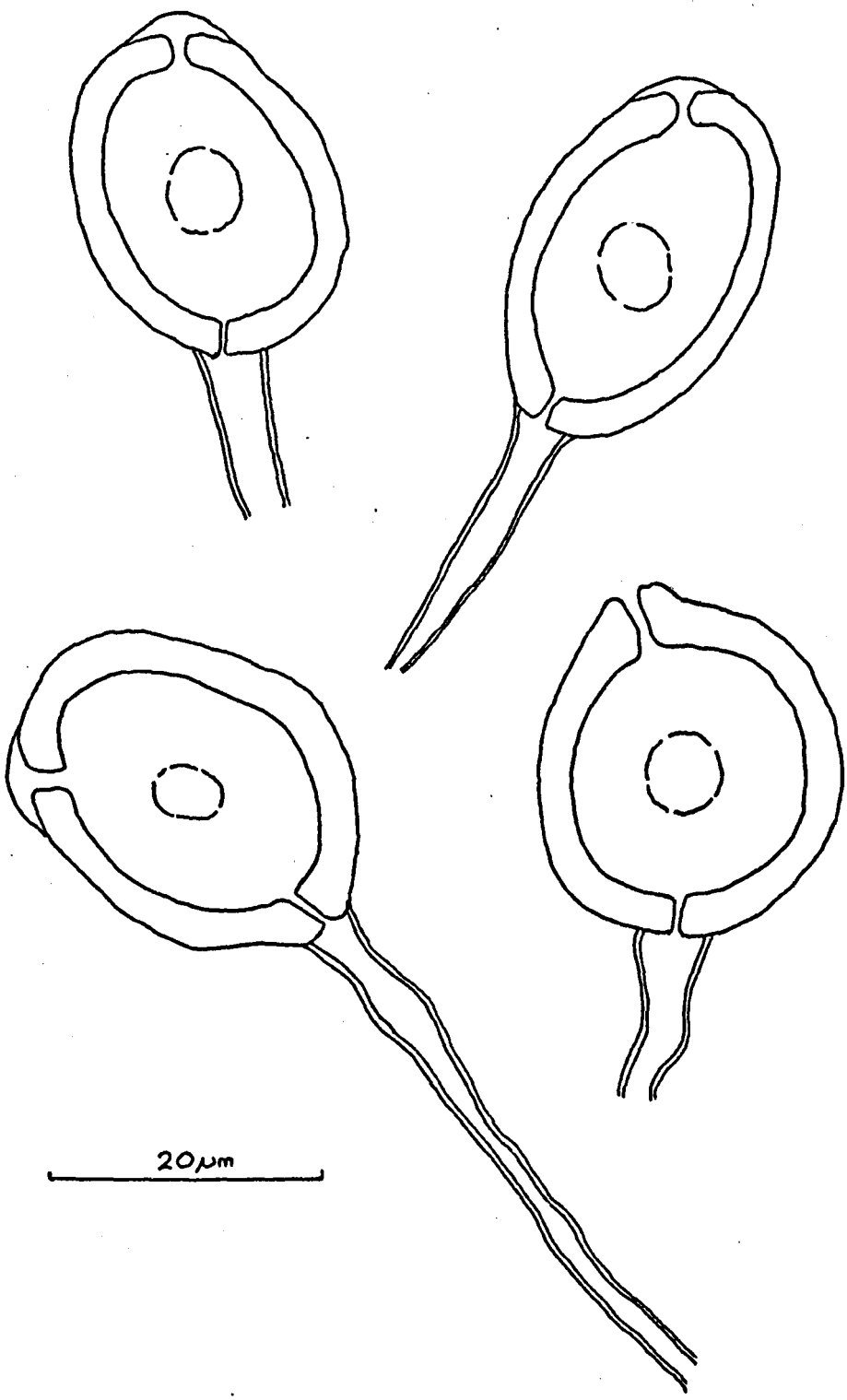


Fig. 2.8. U. dianthi teliospores.
Bodies, believed to be aggregations of lipid vesicles, are visible in the spores.

over the germ-pore; smooth surfaces, pedicel colourless.

2.33 P. arenariae teliospores.

A light microscope was used to examine teliospores of P. arenariae. Usually the spore consisted of two cells, though occasionally one and three celled types were found (Fig. 2.9). The thickest part of the spore wall was always at its rounded or pointed apex. No germ-pore areas were obvious in the spore wall before germination. Hyaline stalk cells were always attached to the spores and were persistent. When mature, the cells of the teliospores could be seperated. In some cases the force of the protruding promycelial growth from the lower germinating cell removed the top cell. Teliospores on D. barbatus were described as:-

Oblong, fusiform, thickened (up to 11 μm) pointed or rounded apices; gently constricted rounded or attenuated bases; smooth, yellowish brown, 30-50 x 13-17 μm; pedicels hyaline persistent, 40-80 μm long.

The teliospores on D. barbatus generally resembled those found on the other hosts of P. arenariae that were used in host range experiments (see Chapter 1.33). However, pedicel length was found to vary slightly, the greatest average lengths being recorded on D. caryophyllus var. Grenadin Scarlet (Fig. 2.10). On S. sublata a much higher proportion than normal of teliospores were one celled. (Fig. 2.11).

2.34 P. arenariae basidiospore production.

Under suitable conditions of temperature and humidity (see Chapter 3.31.1), the teliospores germinated and produced delicate promycelia. If surface water was present on the teliosori, abnormally long promycelia developed (Fig. 2.12a). Under hanging drop conditions, extended promycelial growth occured to give promycelia up to 290 μm in length after 68 h.

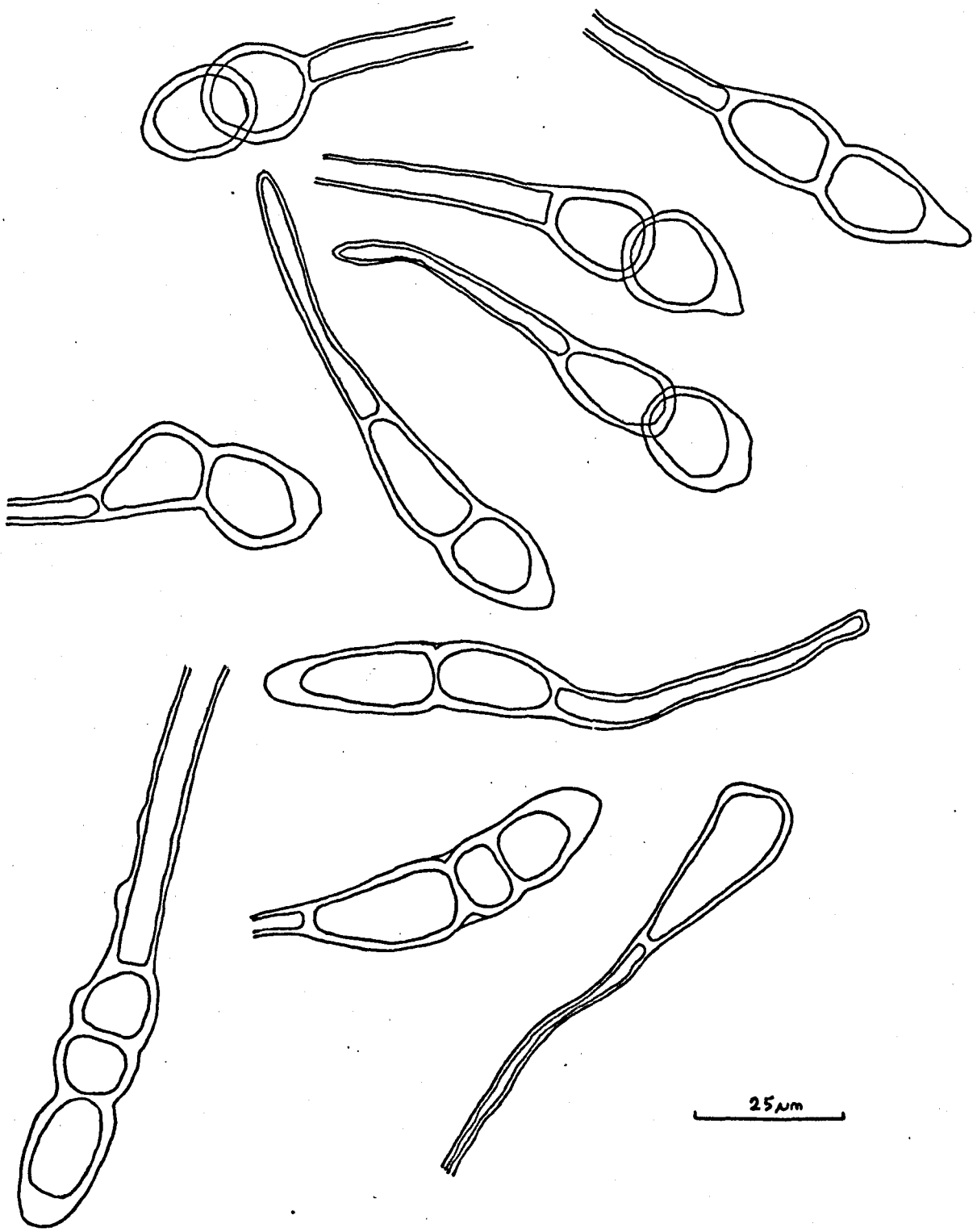


Fig. 2.9. P. arenariae teliospores found on D. barbatus.

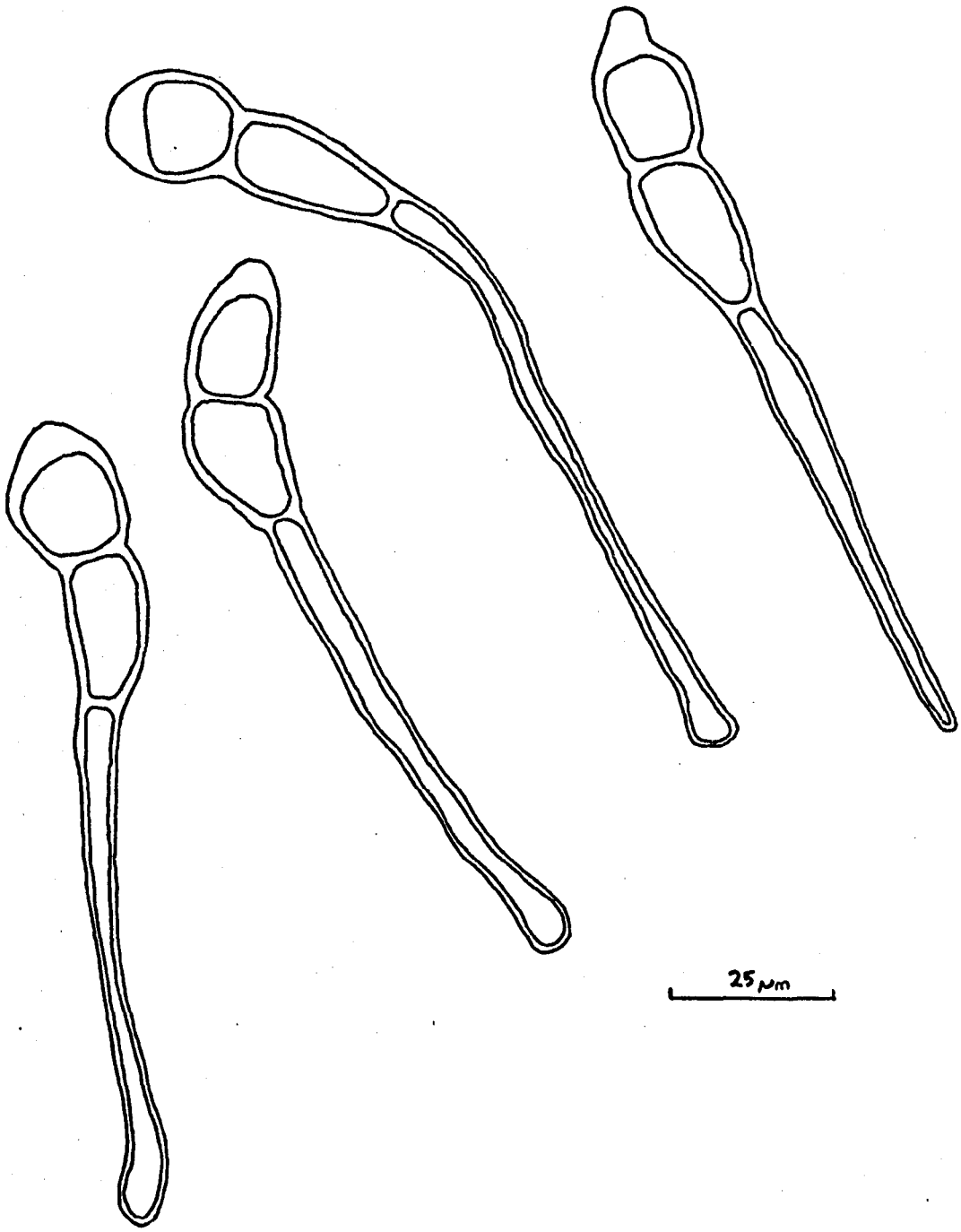


Fig. 2.10. P. arenariae teliospores found on D. caryophyllus.

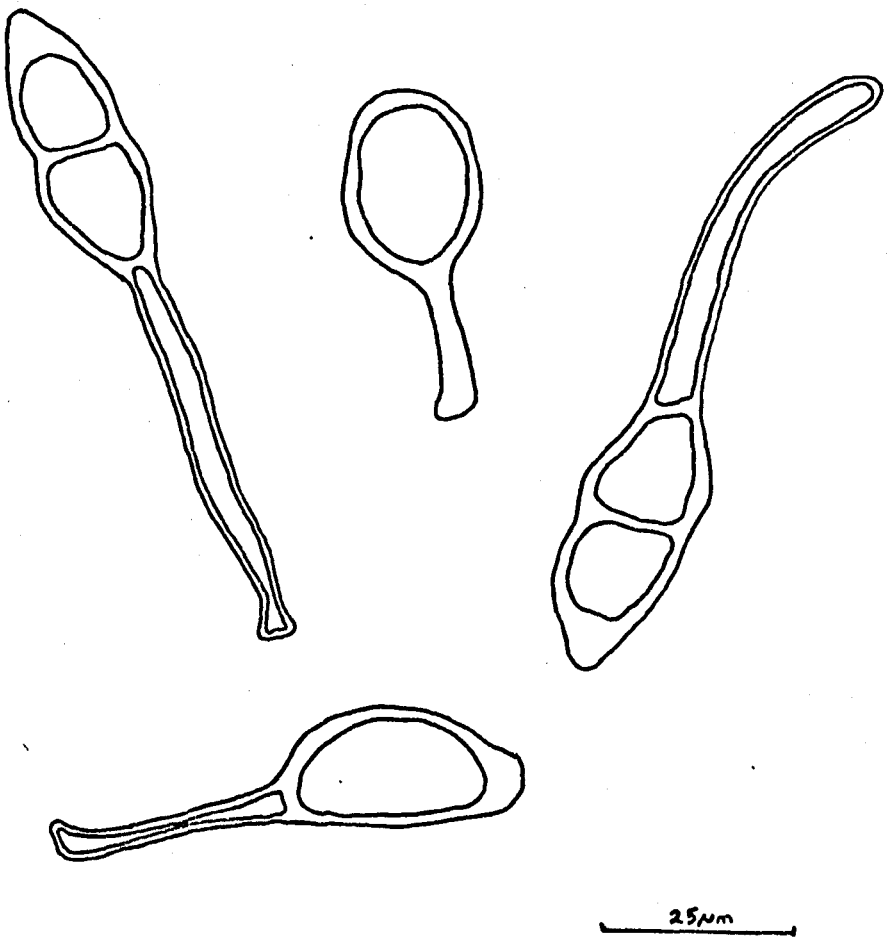


Fig. 2.11. P. arenariae teliospores found on S. sublata.

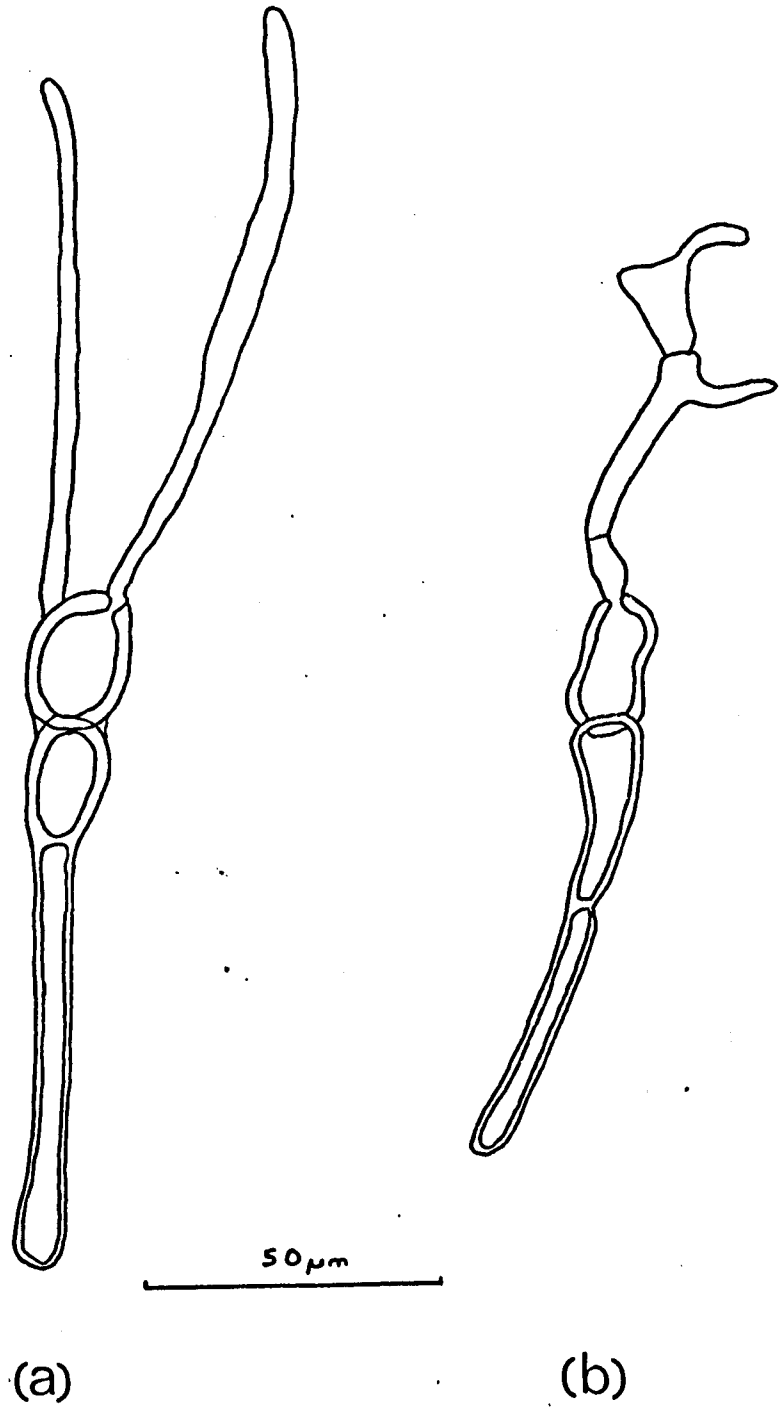


Fig. 2.12a. P. arenariae teliospore germinating in a hanging water drop and developing unusually long and undifferentiated promycelia.

Fig. 2.12b. P. arenariae teliospore with a normally developed two celled basidium (basidiospores discharged).

The average length was found to be about 150 μm. Growth presumably ceased when the food reserves of the teliospore cell were exhausted. The cytoplasm in these promycelia was disorganised and no basidiospores were formed. Arthur (1929) noted this, and in a diagram showed an 'indefinite' and non-septate promycelium containing cytoplasm filled with large vacuoles. It seemed that air was the necessary medium for the normal development of a free upright promycelium of definite length and form.

Teliospore germination was initiated when the promycelium began to grow through the spore wall. Both cells of the spore were capable of germination, and it could proceed in both simultaneously if conditions were suitable. Often, however, it was the top spore-cell which germinated first. Here the promycelium appeared to grow through the apex of the spore. Wilson & Henderson (1966) believed that the greater part of the apex thickening was thrown off during the germination process by the emerging promycelium, but observations indicated penetration by digestion of the spore wall.

After germination, the promycelium emerged and from it a basidium of characteristic two-celled shape developed (Fig. 2.12b). Great difficulty was experienced in the preparation of slides which showed basidiospores in position on sterigmata. The inevitable agitation that occured during mounting usually detached them. However, the camera lucida drawings that were made showed the stages in the development of a basidiospore (Fig. 2.13).

2.35 P. arenariae basidiospores.

2.35.1 Structure and nuclear content.

The dimensions of the typically ovoid to hemispherical

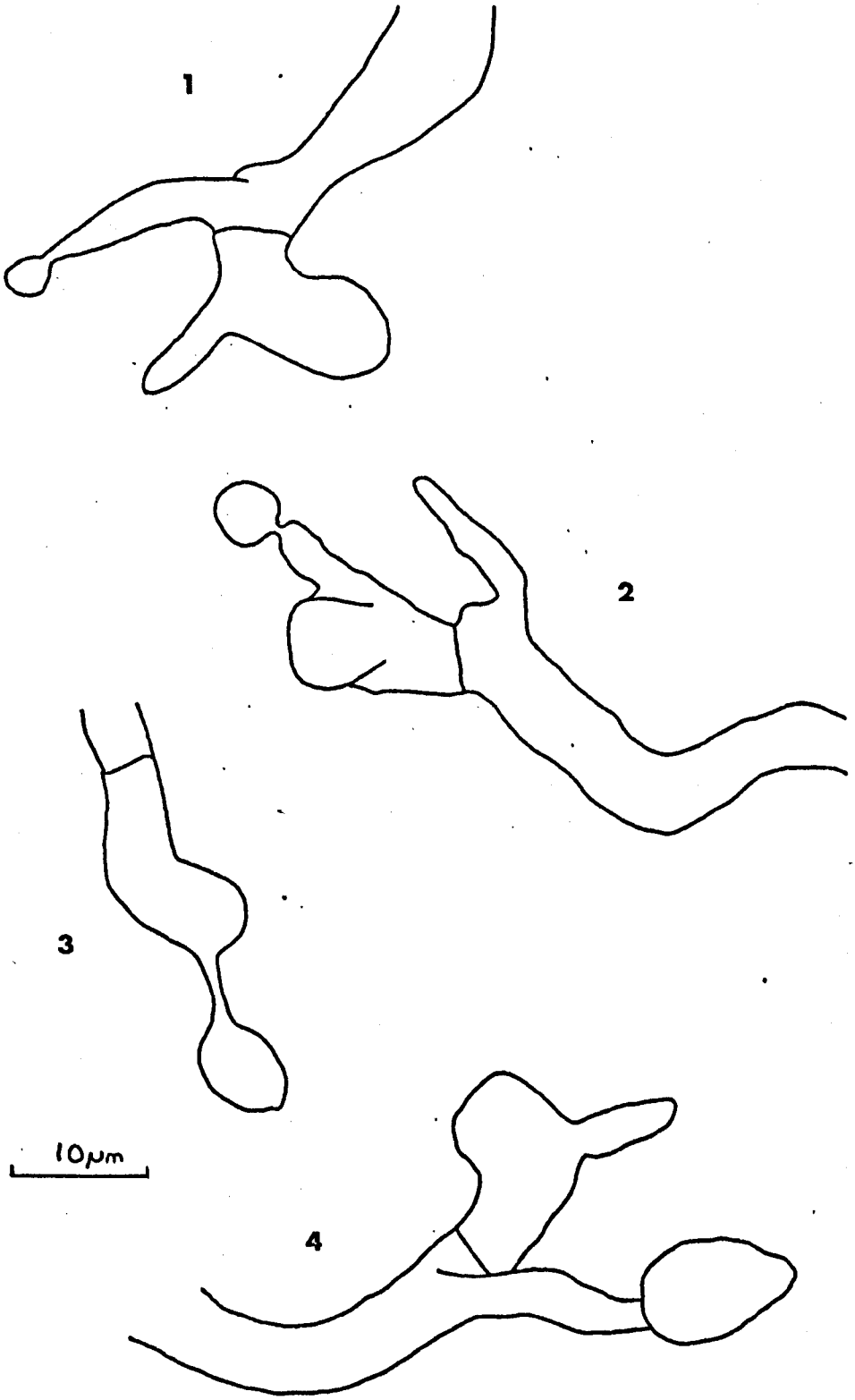


Fig. 2.13. Stages in the development of the basidiospore on the basidium (formed on the germination of a P. arenariae teliospore).

basidiospores were 12-16 x 7-9 µm. A slight swelling of the spores seemed to occur during the germination processes, which was attributed to water uptake. The sporidia were usually one-celled but occasionally two and three-celled variations were found. The spores seemed able to germinate almost immediately and could produce long germ-tubes. Two-celled basidiospores were found with two germ-tubes, one originating from each cell (Fig. 2.14). On water agar the germ-tubes could grow to around 30 µm in length.

Lindfors (1924) investigated the movements of nuclear material during basidiospore development. The migration of a diploid nucleus from a teliospore, cell into a developing basidium was observed. In the basidium the nucleus divided, the division being followed by the appearance of a cross wall. Meiosis was completed by a second division, which he found was not followed by the appearance of another cross wall. The final situation was therefore two nuclear pairs in the young basidiospore. Consequently Lindfors stated that the total mycelium of P. arenariae was dikaryotic.

Anatomical observations confirmed that only one cross wall and two basidiospores were formed per basidium but great difficulty was experienced in tracing the movements of the nuclei. The nuclear material in the mature spores, however stained very successfully.

The ungerminated spore usually contained two nuclei which increased to four on germination (Fig. 2.15), sometimes before (Pl. 2.7). After germination, the four nuclei migrated from the basidiospore into the germ-tube (Fig. 2.16).

2.35.2 Germ-tubes and infection structures.

Appressoria were often formed very soon after germination (Fig. 2.17). Germ-tubes could however extend some way over the leaf surface before appressorial formation (Fig. 2.18).

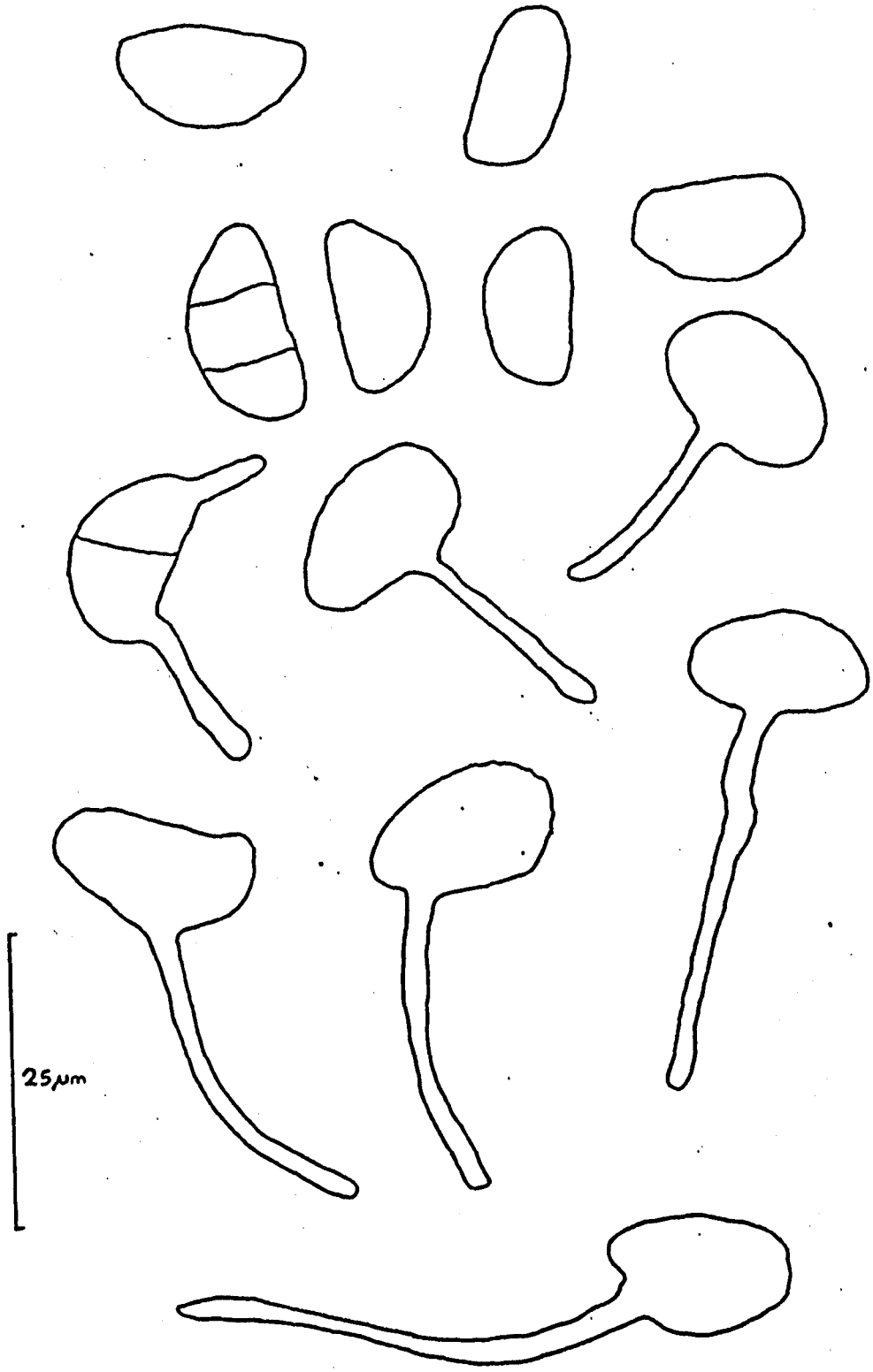


Fig. 2.14. P. arenariae basidiospores.

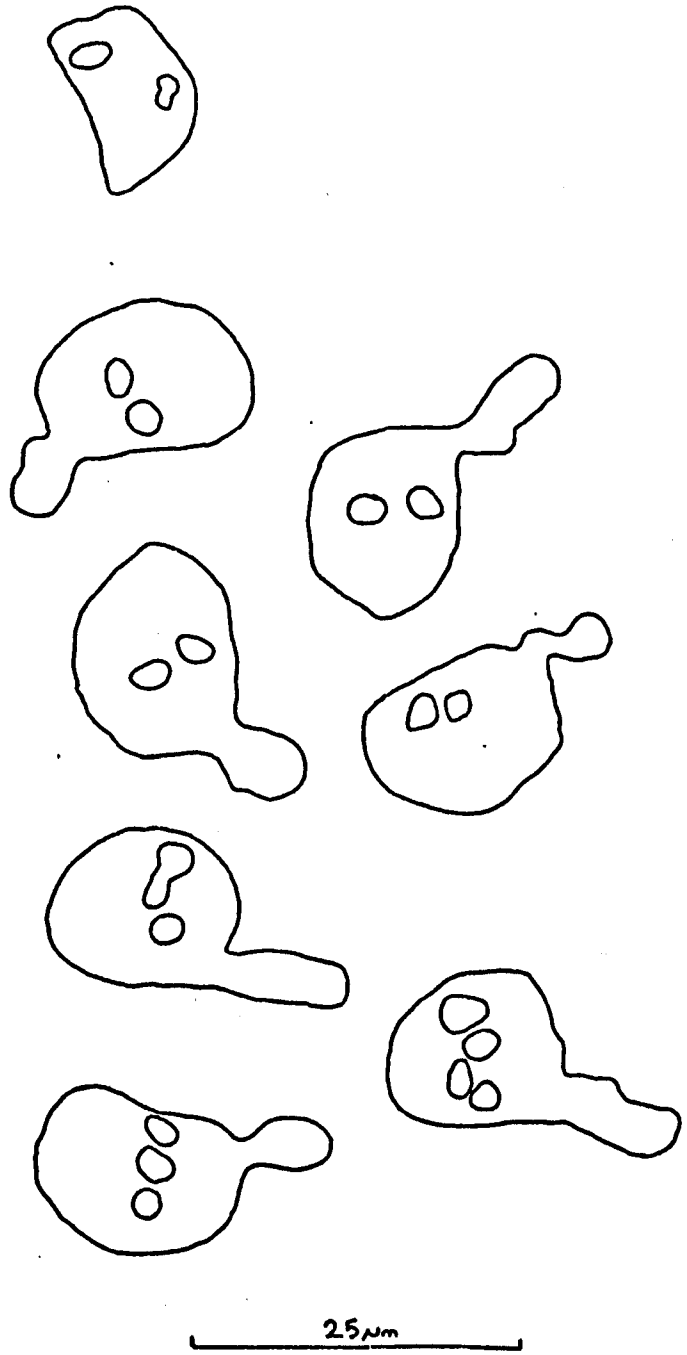


Fig. 2.15. Germinating P. arenariae basidiospores showing nuclei.

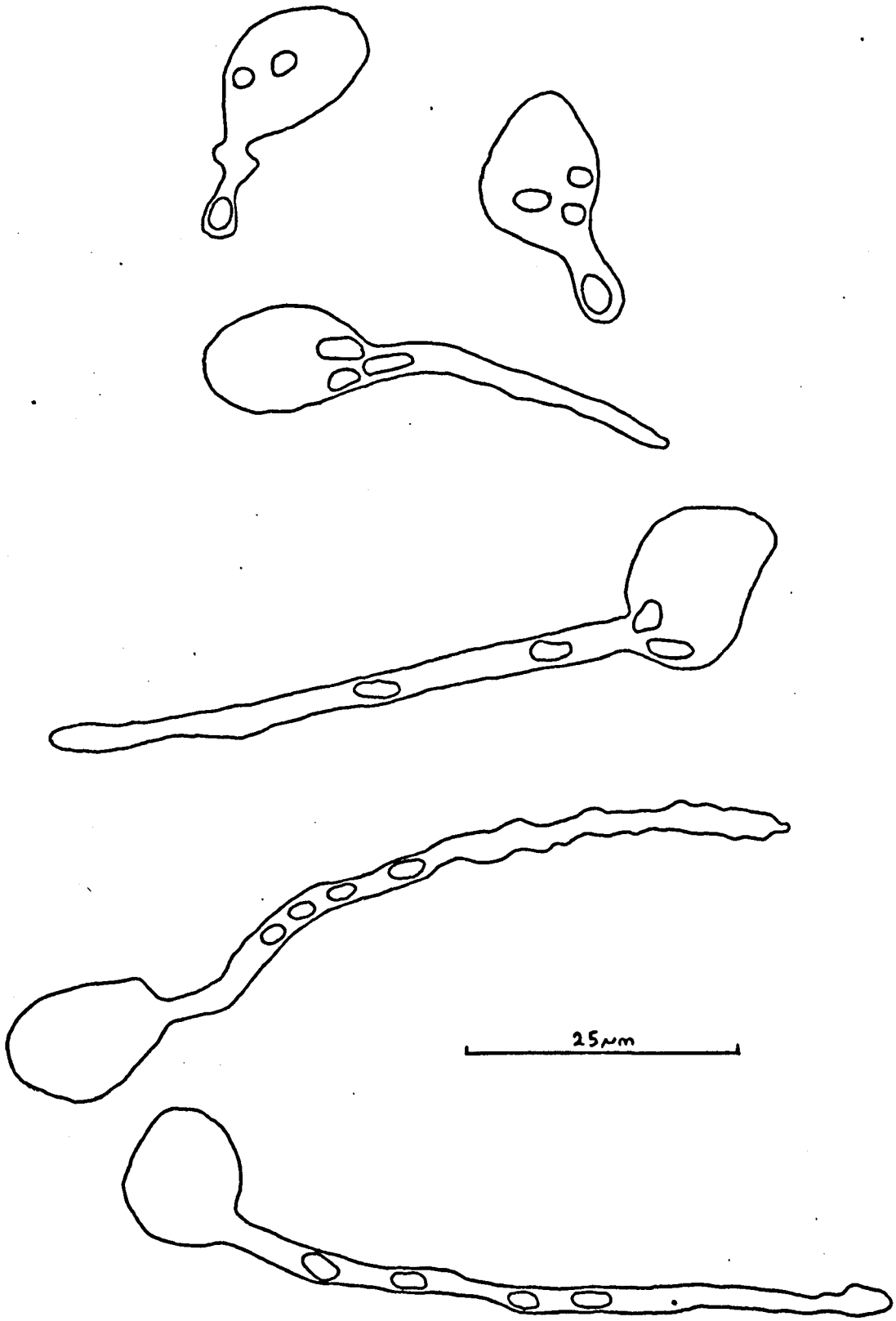


Fig. 2.16. Germinating P. arenariae basidiospores showing the migration of nuclei to the germ-tubes.

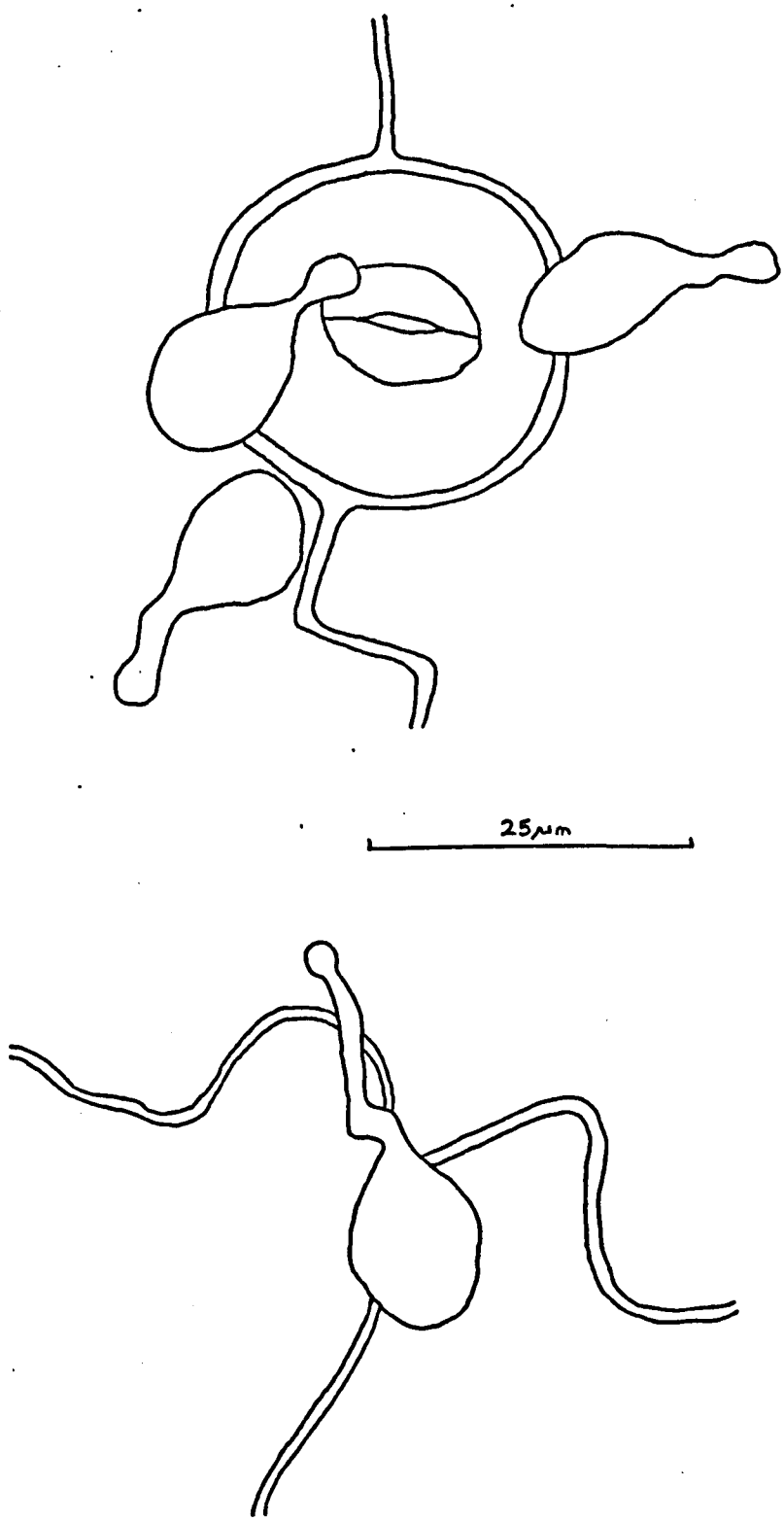


Fig. 2.17. P. arenariae basidiospores germinating and forming appressoria on D. barbatus leaf surfaces.

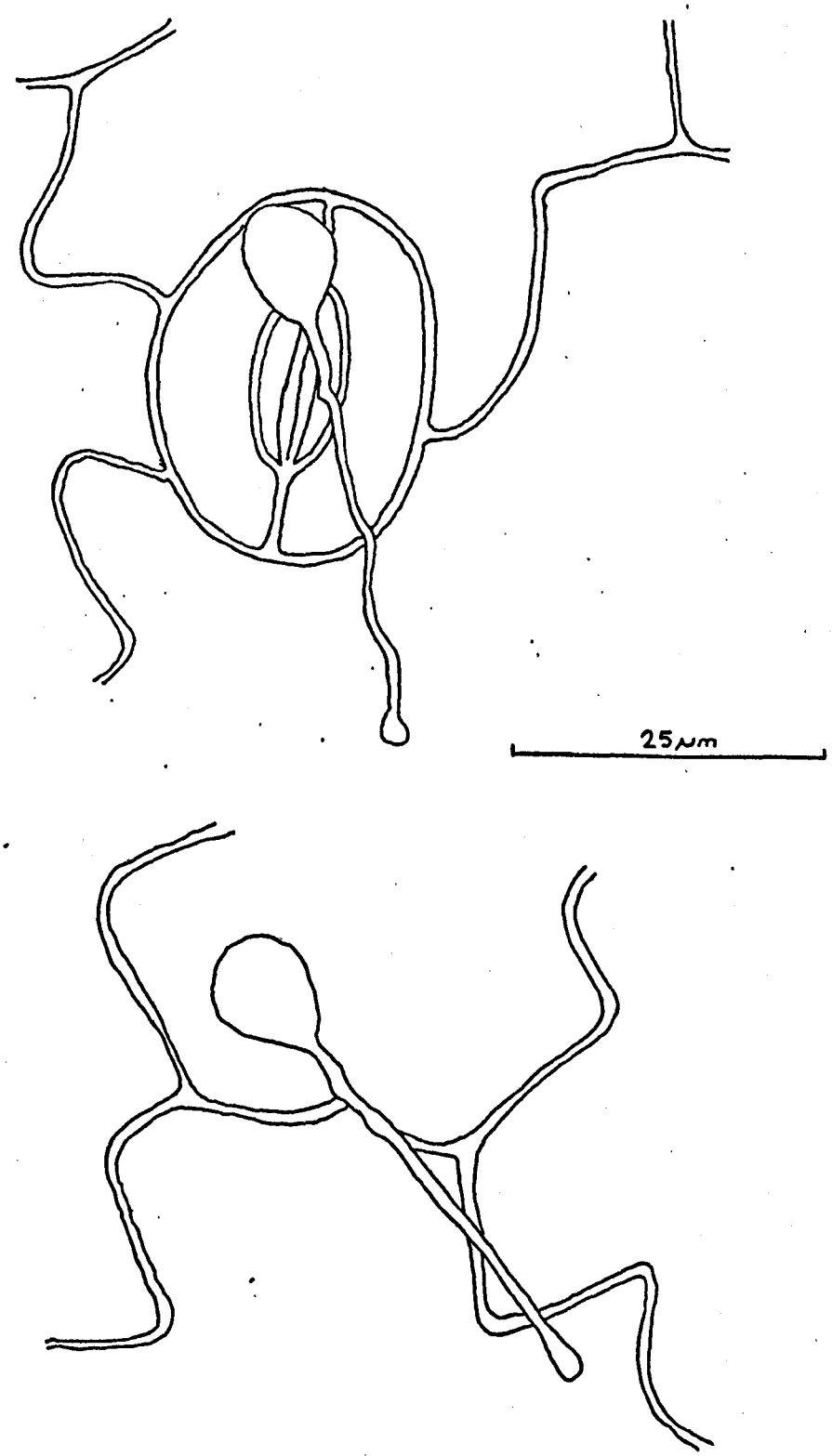


Fig. 2.18. P. arenariae basidiospores with germ-tubes and appressoria on D. barbatus leaf surfaces.

The diameter of germ-tubes varied from 0.5-2.5 µm, and lengths of up to 40 µm were recorded. From the appressoria, narrow infection hyphae penetrated the cuticle and epidermal cell wall.

2.4 DISCUSSION

No significant differences were revealed by the slight variations in U. dianthi urediospore dimensions. An important item however, that up to five germ-pores were observed per urediospore and not four as in the latest C.M.I. description (Punithalingam, 1968).

The spinal ornamentation on U. dianthi urediospores could possibly be used for diagnostic purposes if the 'two spines surrounded by a common ridge' (Pl. 2.6) proved to be characteristic of the species. No other record of this feature has been published. It was thought unlikely that spine and ridge dimensions would be useful as they varied considerably and resembled those found on other species.

The fact that germ-pores were indistinguishable from the rest of the spore surface, meant that germ-tubes must have emerged from the spore in areas that possessed spines. The absence of cracks or splits in the spore wall on germination indicated that the germ-tube passed through the wall along a defined germ-pore channel. It is probable that the spore absorbed water through the germ-pores which as well as activating the spore cytoplasm loosened the 'plug' in the pore channel. Usually only one germ-tube is observed leaving the spore, although there can be from 2-5 germ-pores. This indicated that the 'loosening process' was gradual and differential, the first 'plug' succumbing to the pressure of the swelling spore cytoplasm being the exit channel.

On carnation leaves appressoria were only formed over stomata. On D. barbatus, the germ-tubes of U. dianthi urediospores behaved erratically. Appressoria that were formed were usually not positioned over stomata. This was believed to be one of the reasons for poor infection results with Sweet Williams. It may have been due to the importance of cuticular ridges in directional growth of the germ-tube (Maheshwari & Hildebrandt, 1967) or the action of unfavourable leaf diffusates.

The description of U. dianthi teliospores closely resembles that of the Commonwealth Mycological Institute (Punithalingam, 1968). The spore walls however appeared smooth when viewed through a light microscope. No evidence of rounded warts on the surface was found.

The P. arenariae teliospore dimensions were also similar to previous descriptions. Variations of some spore characters (e.g. pedicel length and proportions of one celled spore types to two) on some hosts were believed to have been partly responsible in the past for the classification of P. arenariae as a number of different species.

The process of basidiospore production was found to be identical with that proposed by Lindfors (1924). A two-celled basidium was formed on germination of a teliospore and one basidiospore was produced per cell. From studies on the genetic behaviour of P. arenariae it seems that its nuclear cycle can be summarised as in Fig. 2.19.

The longest basidiospore germ-tube on water agar was found to be 30 μm in length. On the leaf surface, lengths of 40 μm were recorded. It is possible that leaf exudates provide nutrients to the germ-tube cytoplasm enabling it to survive and grow longer.

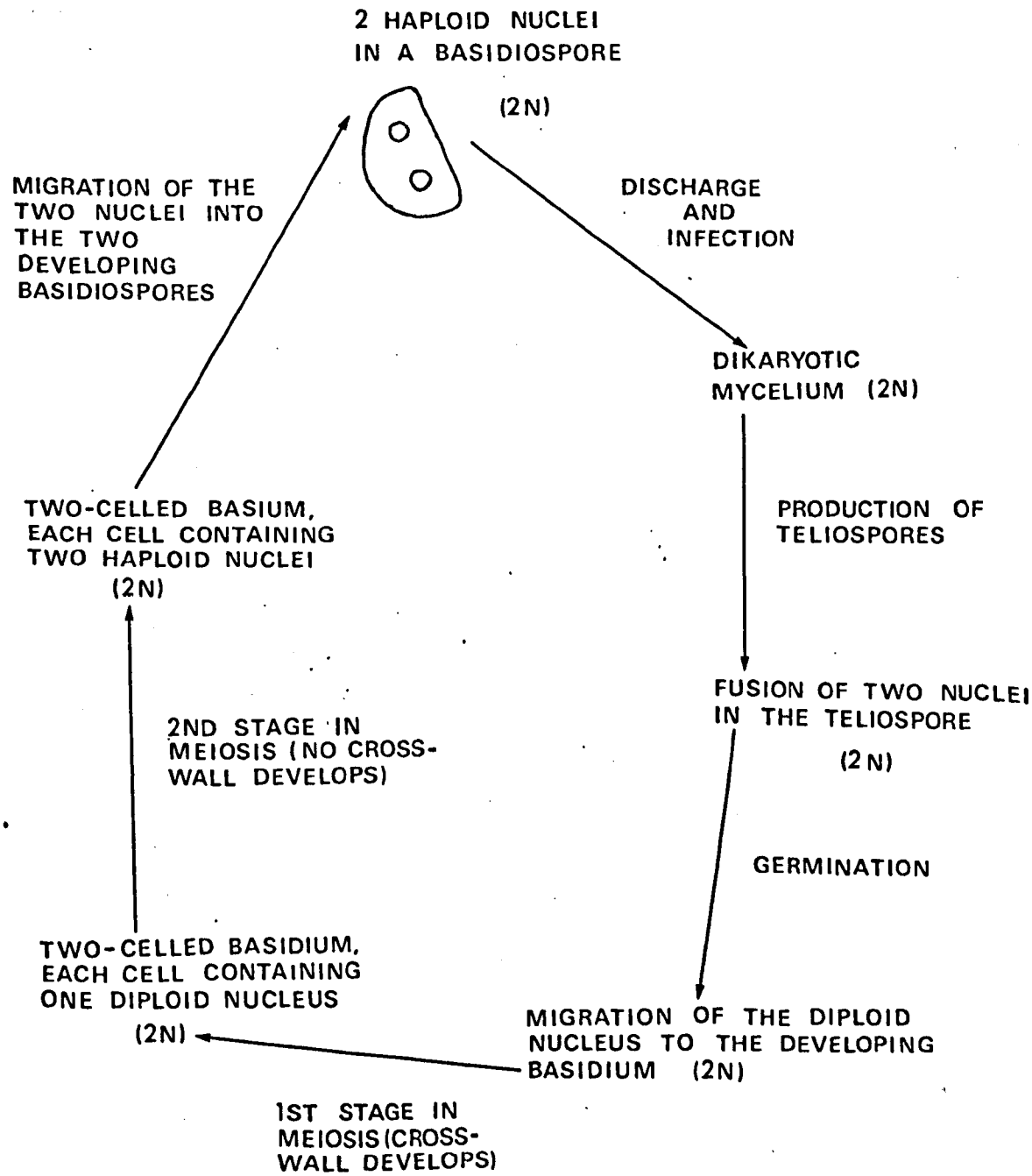


Fig. 2.19. The nuclear cycle of P. arenariae.

CHAPTER 3 SPORE PHYSIOLOGY.

3.1 INTRODUCTION

The work in this section was done using the urediospores of *U. dianthi*, except that the optimum germination temperature for the teliospores of *P. arenariae* was determined for use in later experiments.

The physiology of resting and germinating urediospores has been reviewed by Mains (1917), Arthur (1929), and more recently by Allen (1959), Cochrane (1958, 1960), Shaw (1964) and Staples & Wynn (1965).

In general the optimum temperature for germination of urediospores of different species is between 12 and 23°C and the optimum pH in the range 4.5 - 6.5 (Arthur, 1929; Stock, 1931). Doran (1919) working with *U. caryophyllinus* (*U. dianthi*) in the U.S.A. found that the minimum, optimum and maximum temperatures for germination of urediospores were 4°C, 14°C and 29°C. Székely (1967) found that *U. dianthi* urediospores in Rumania germinated only in the presence of water droplets at a mimimum temperature of 2°C, a maximum of 35°C and an optimum of 24°C.

Germination tests were undertaken with *U. dianthi* urediospores harvested from *D. caryophyllus* var Grenadin Scarlet plants grown in the glass-house. Besides spore germination, the length of urediospore germ-tubes was taken as a determining factor in indicating the optimum germination conditions (Johnson, 1912).

In a number of species of rust fungi there occurs a phenomenon called self inhibition (Allen, 1955; French, Massey & Weintraub, 1957). It was found that in dilute phosphate buffer, at or below pH 7.0 urediospores of *Puccinia graminis* f. sp. *tritici* produced, within 8 and 24 h under aerobic

conditions, one or more substances highly active in preventing germination. At pH 6.0 but not at pH 7.3, the activity of crude preparations containing the inhibitors was reduced by aeration. The inhibitors were still effective after exposure to 100°C in sealed containers, but their activity was lost after exposure to glass surfaces. Concentrations of the inhibitors, which were found to retard elongation of germ-tubes, still allowed spores floating on aqueous solution to develop infection structures. The active substances were readily removed from water by distillation and retained their activity against spores after diffusion across an air gap.

Evidence was obtained by Forsyth (1955) that the active substance was 2 - methyl - 2 - butene (trimethylethylene). However, the self inhibitors that prevented germination of Uromyces phaseoli urediospores were claimed by Wilson (1958) to be quite different. They could be collected on filter paper in water containing urediospores or in agar films above and in contact with rust pustules. Water extracts of urediospores were found to contain at least eight ninhydrin positive substances, the predominant ones being beta - alanine, glutamic, and aspartic acid. The acids which prevented germination at 100 p.p.m., were present in sufficient concentration to account for inhibition of germination in dense spore suspensions. Bell & Daly (1962) could not confirm Wilson's findings. They found that in neutral solutions aspartic and glutamic acids were not inhibitory, even at concentrations of 1000 p.p.m. They considered that the inhibitor was produced by a metabolic breakdown of other substances.

Hassebrauk & Schroeder (1964) found some evidence that the inhibitors of P. striiformis urediospores were short chain fatty acids, or their derivatives.

Allen, Strange & Elnaghy (1971) thought that inhibitors in P. graminis f. sp. tritici urediospores might be interconvertible phenolic compounds. Macko, Staples, Allen & Renwick (1971) identified them as the cis and trans isomers of methyl 4 - hydroxy - 3 methoxycinnamate (methyl ferulate). The urediospore germination inhibitors in bean rust, snapdragon rust and sunflower rust, have been identified as the cis and trans isomers of methyl 3, 4 - dimethoxycinnamate (Macko, Staples, Gershon & Renwick, 1970; Macko et al., 1971).

In this study, exudates from U. dianthi urediospores were tested for inhibitory action.

3.2 MATERIALS AND METHODS.

3.21 Collection of P. arenariae teliospores.

Diseased D. barbatus var. Scarlet Beauty leaves with young teliosori were collected from infected plants in the glass-house. Great care was taken to ensure that the spores had not germinated or were not germinating. The material was used in the experiment as soon after gathering as was possible.

3.22 Collection of U. dianthi urediospores.

Urediospores were brushed from mature open sori of approximately the same age and location into plastic Petri dishes using a small camel hair paint brush. The spores were stored at 4°C in plastic Petri dishes sealed with adhesive tape, and used soon after collection, usually within 24 h.

3.23 Germination and germ-tube length assessment.

The criterion taken for germination was the emergence of a germ-tube to a length exceeding its width. U. dianthi urediospores always gathered in a roughly uniform group in the lowest point of a hanging drop, and in the centre of the solution in a staining block. In each field of microscope vision,

the first 100 spores encountered in a systematic observation procedure were counted and germination numbers noted. Where 200 spores were counted, two random fields of vision were observed, where 500 spores, five random fields of vision.

Germ-tube legths were measured with the aid of a micrometer eye piece. Coiling of some long germ-tubes made their measurement difficult. These difficult lengths were estimated. All germ-tubes encountered in systematic searches across random fields of microscope vision were measured until the total number of germ-tube lengths required were recorded.

3.24 Analysis of germination results.

The results in each experiment involving urediospore germination were analysed statistically using Student's 't'-test. When the results involved more than a simple comparison between two means, each mean was paired and compared with each of the others and the levels of significance noted.

Most differences between means were found to be significant at the 5% level.

3.3 RESULTS

3.31 Germination and temperature.

3.31.1 The effect of temperature on the germination of P. arenariae teliospores.

Experimental procedure. In this experiment the conditions closely matched those found in vivo, and involved measuring the germination of teliospores at various temperatures at 100% r.h.

The humidity vessel chosen for these experiments was a small staining block, which could be easily sealed using petroleum jelly and thick glass cover-plates. A little distilled water was placed in the bottom of the staining block,

and covered by a small square glass cover-slip which was held above the surface by the curved sides of the block. Pieces of teliosori, containing aggregations of teliospores, were then positioned on the cover-slips and the block sealed (Fig. 3.1). The blocks were then placed in the incubators. The experiment had one replicate.

After 24 h when the experiment was terminated, the teliosori pieces were removed from the cover-slips, and teased apart in lactophenol blue stain on clean glass slides. From each block, two slides were prepared, Therefore, for each temperature, there were four slides. On each slide the first fifty morphologically mature two-celled teliospores observed were used to determine percentage germination. The percentages were averaged for each temperature.

Results. The results indicated an optimum germination temperature of around 20°C. (Table 3.1, Fig. 3.2). Although teliospores germinated at 4°C, 26°C, 30°C and 35.4°C, growth of the promycelium was restricted and abnormal, and no basidiospores were produced.

3.3.2 The effect of temperature on U. dianthi urediospore germination.

Experimental procedure. Urediospore germination was estimated using the hanging drop method. Suspensions of urediospores could not be formed because of their hydrophobic properties. Spores were therefore floated on distilled water (1°C pH 5.3) in a small glass staining block. The spores were mixed to ensure an even surface distribution. Single drops containing spores were hand pipetted from the liquid surface with an eye lotion dropper, and placed on glass cover-slips. Each drop was approximately the same size and contained about 1000 spores. The cover-slips were secured with petroleum

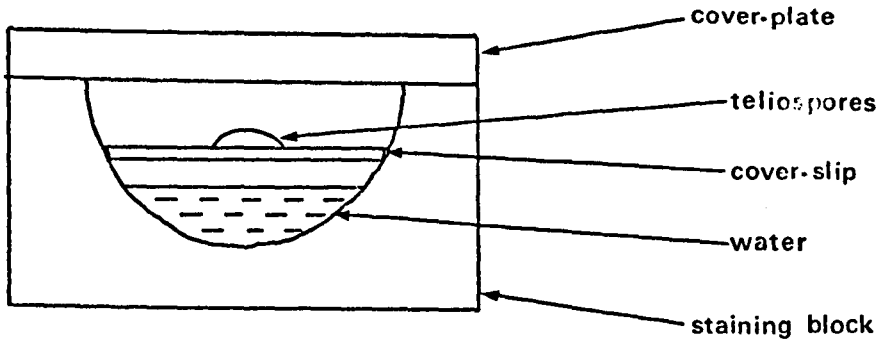


Fig. 3.1. Humidity vessel used in assessing P. arenariae teliospore germination.

Fig. 3.2. (see over, page 63).

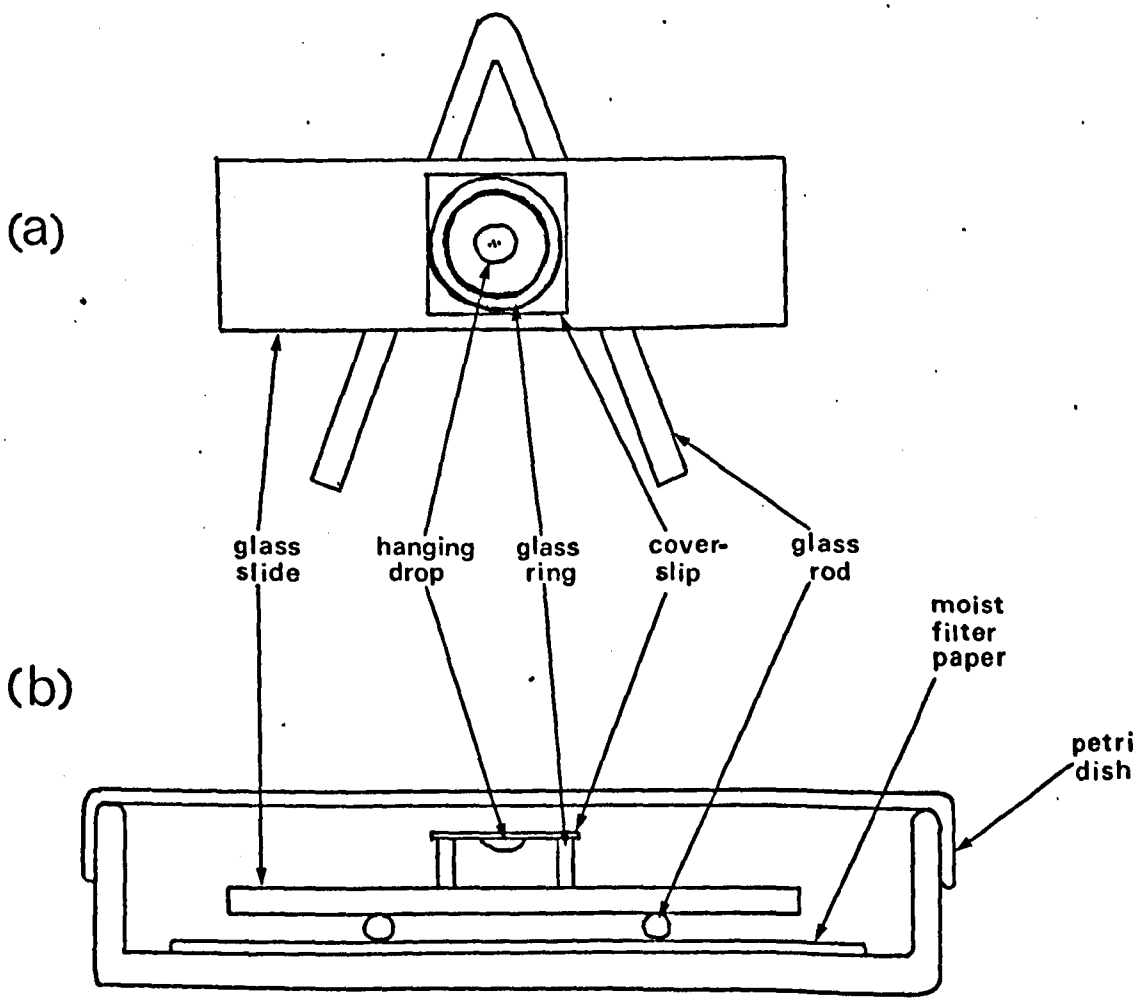


Fig. 3.3. Apparatus used in the hanging drop method of assessing U. dianthi urediospore germination, (a) viewed from above, (b) viewed from the side.

Fig. 3.2. The effect of temperature on P. arenariae teliospore germination.

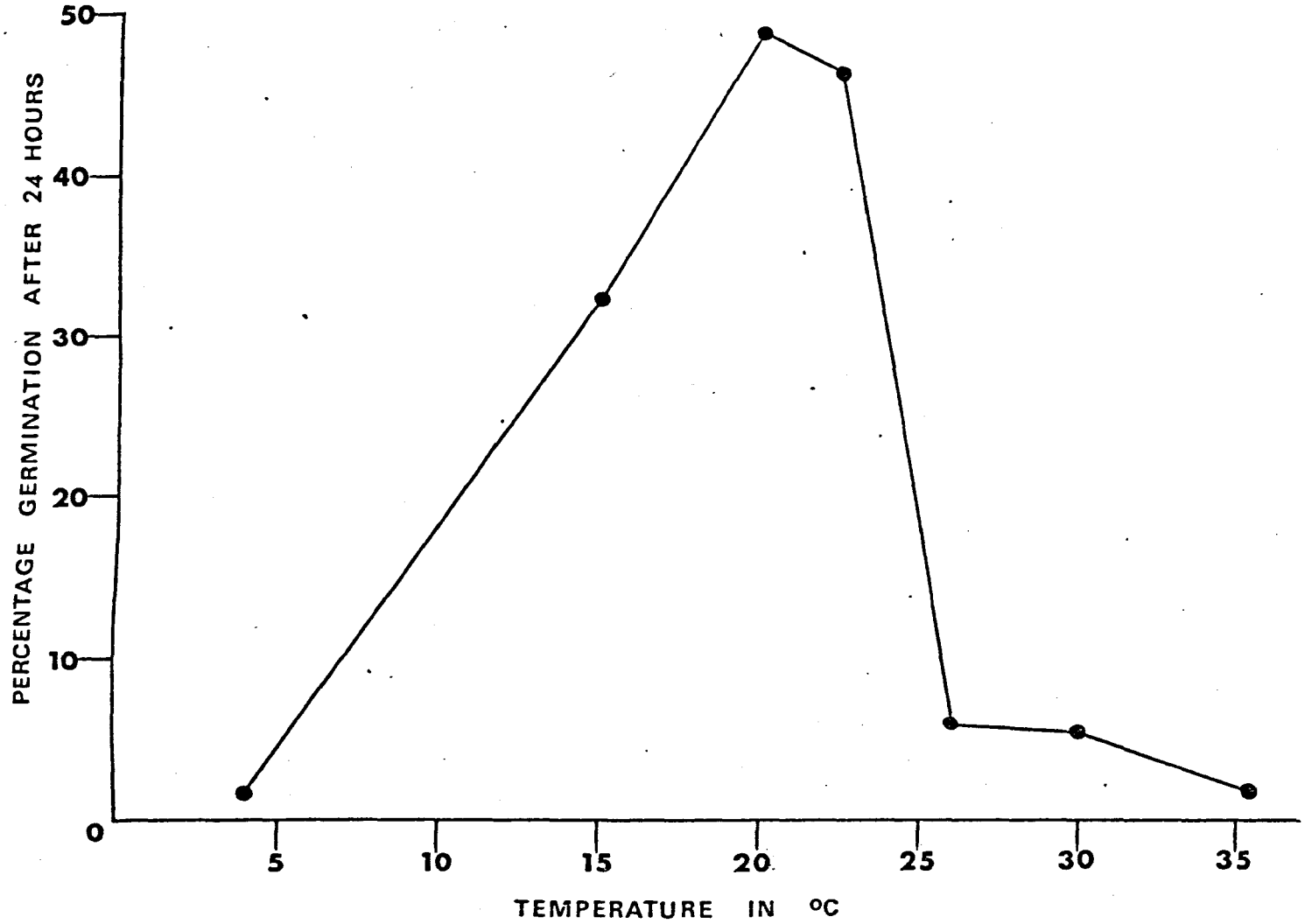


TABLE 3.1. The effect of temperature (4-35.5°C) on P. arenariae teliospore germination.

Temperature °C	% Germination after 24 h
4	1.5
15	32.5
20	49.0
22.5	46.5
26	6.0
30	5.5
35.5	2.0

TABLE 3.2 Percentage germination of U. dianthi urediospores at various temperatures and times.

Time in h	Temperature °C						
	1	5	10	15	20	25	30
1	-	-	-	23.8	30.5	33.5	8.5
2	-	-	17.3	52.2	65.7	51.0	25.5
4	-	8.8	48.2	63.7	73.3	63.7	38.8
8	1	24.3	53.2	75.7	80.0	70.2	43.5
12	2.8	28.8	59.2	77.0	83.8	74.7	43.8

TABLE 3.3 The effect of temperature (1-30°C on U. dianthi urediospore germ-tube length. Measurements in µm after 11 h.

Temperature °C						
1	5	10	15	20	25	30
35.8	93.2	207.1	318.9	382.1	279.7	11.8

jelly) and inverted over glass rings which were fixed to slides with Neutral Canada Balsam. The slides were placed on bent glass rods in glass Petri dishes in incubators (Fig. 3.3). The experiments were performed in triplicate. Two hundred spores observed in each drop were used to assess germination and between 50 - 70 spores in one drop per temperature to assess germ-tube length.

In the first experiment (temperature range 1°C - 30°C) germination readings were taken after 1, 2, 4, 8 and 12 h and germ-tube length estimated after 11 h. In the second experiment (temperature range 17.5°C - 25°C) germination readings were taken after 1, 3, 7 and 11 h and germ-tube length estimated after 2, 4, 6, 8, 10, 12, and 24 h.

Results. Experiment 1. A germination optimum of around 20°C was indicated (Table 3.2 and 3.3, Fig. 3.4). Although germination percentages at 30°C were relatively high, germ-tube lengths were very low. At this temperature infections could not have occurred.

No statistically significant differences were found at 1 h, 8 h and 12 h, between the germination readings at 15°C and 20°C.

Experiment 2. Statistically no significant difference in germination was found between any of the readings at any of the temperatures. Germ-tube length might therefore be considered to be important in determining the optimum temperature for germination. Using this criterion, an optimum germination temperature of 20 - 22.5°C was indicated (Table 3.4 and 3.5, Fig. 3.5).

3.32 The effect of pH on U. dianthi urediospore germination.

The effect of pH on urediospore germination is complicated by the fact that the experiments are generally carried out in

Fig. 3.4. The effect of temperature (1-30°C) on U. dianthi urediospore germination (●———●) and germ-tube length (o- - - -o).

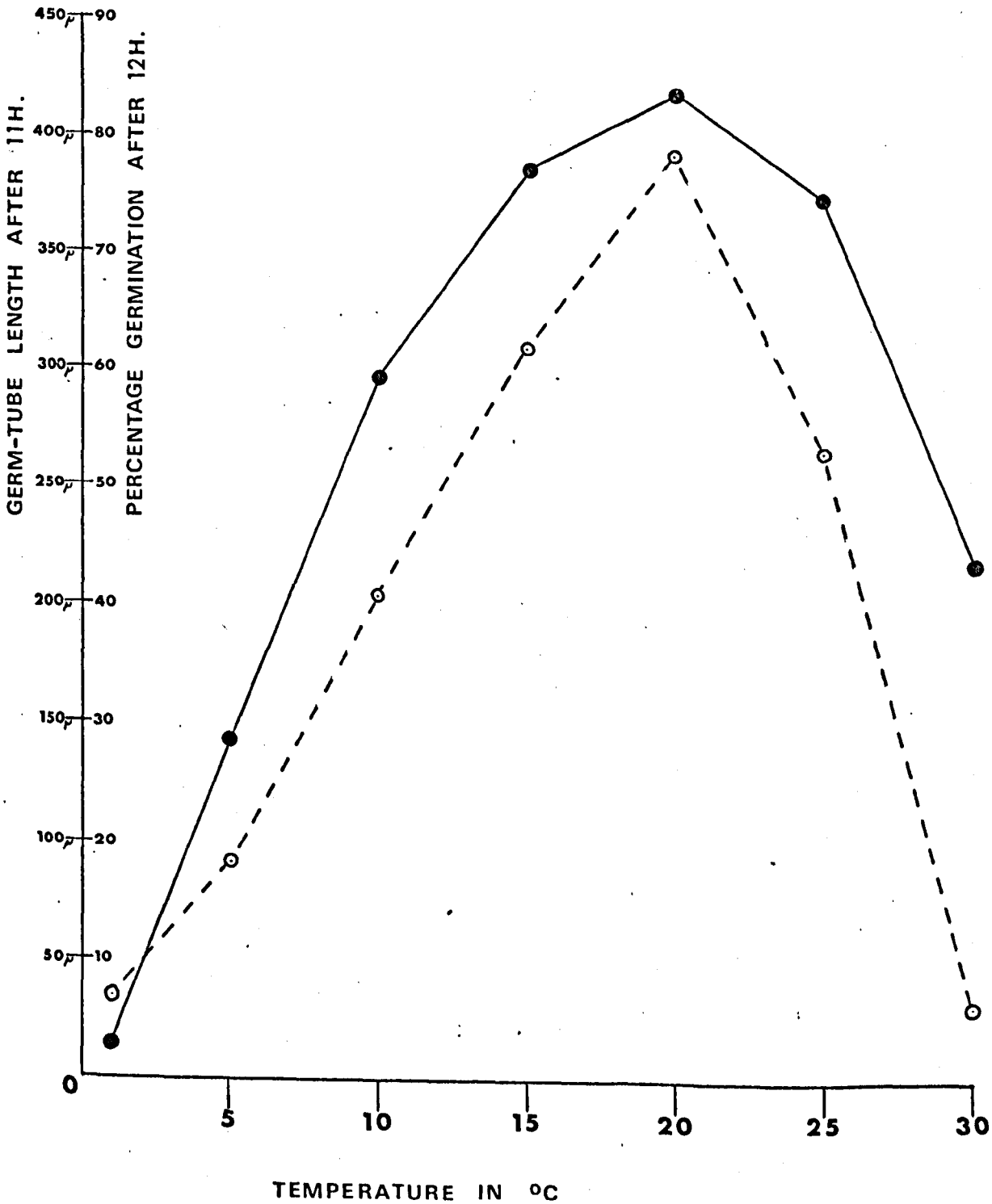


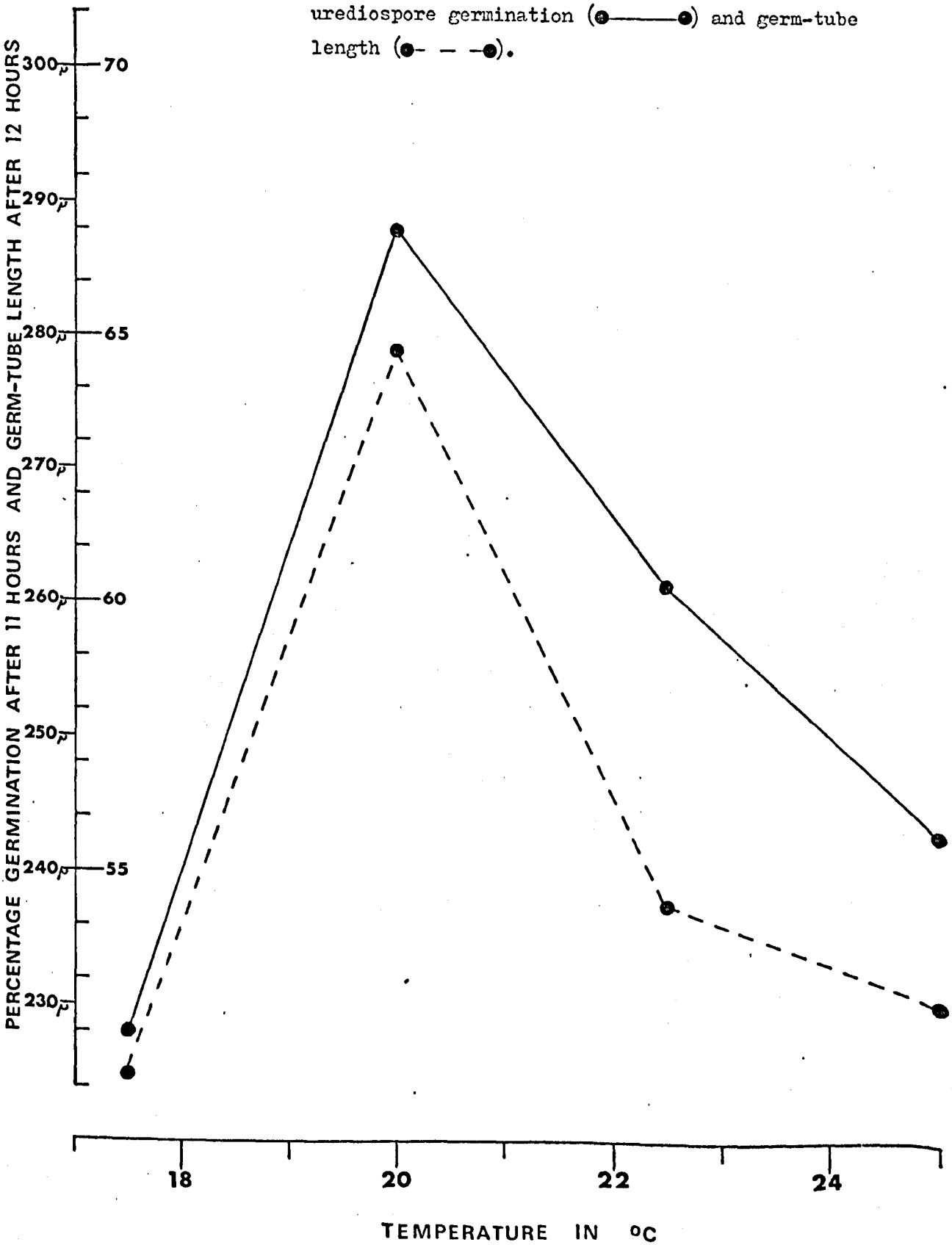
TABLE 3.4 The effect of temperature (17.5 - 25°C) on the percentage germination of U. dianthi urediospores.

Time in h	Temperature °C			
	17.5	20	22.5	25
1	11.3	14.3	16.7	12.7
3	36.3	40.3	41.0	32.0
7	45.0	56.7	55.0	51.3
11	52.0	67.0	60.3	55.7

TABLE 3.5 The effect of temperature (17.5 - 25°C) on U. dianthi urediospore germ-tube length. Measurements in µm.

Time in h	Temperature °C			
	17.5	20	22.5	25
2	37.8	39.2	46.2	42.2
4	98.2	136.1	114.9	96.4
6.	154.1	186.7	177.7	168.5
8	185.5	235.5	199.6	185.8
10	204.4	270.9	218.6	197.1
12	225.2	278.9	237.6	230.2
24	382.8	375.9	269.9	232.4

Fig. 3.5. The effect of temperature (17.5-25°C) on U. dianthi urediospore germination (●———●) and germ-tube length (●- - -●).



buffered solutions and it is uncertain whether the result is due to pH or the chemical constitution of the buffer solution. To try to eliminate this difficulty two buffer systems were used.

(a) McIlvaine's Buffer (Na_2HPO_4 - Citric acid)

(b) Sörensen's Buffer (Na_2HPO_4 - KH_2PO_4)

Experimental procedure. Buffer solutions (o.1 M) of various pH values were made up from tables (Vogel, 1951). Final pH values were found by the use of a pH meter. The buffers were sterilised by autoclaving, their pH values rechecked, and then 1.5 ml pipetted into small glass staining blocks. Roughly equal amounts of urediospores were transferred to the surface of the solutions by means of a small camel hair brush. It was assumed that because of dilution by the solutions, the effect of any inhibitors released from the relatively small numbers of spores would be negligible. The glass cover-plates of the staining blocks were secured with petroleum jelly. They were placed in a 20°C incubator.

The experiments were performed in triplicate. Six hundred spores observed for each pH value (200 per block) were used to assess the germination percentage after 4, 8, 12 and 24 h. After 30 h the average germ-tube lengths for each pH value was assessed from between 100 and 120 measurements in one block.

Results. With McIlvaine's Buffer, a germination optimum of around pH 5.15 was indicated. (Table 3.6 and 3.7, Fig. 3.6). There was however no statistically significant difference between the germination readings taken at 8 h and 12 h on the buffer solutions of pH 4.10 and pH 5.15.

TABLE 3.6 The effect of pH (McIlvaine's Buffer) on urediospore germination.

Time in h	Germination Percentages					
	pH					
	3.10	4.10	5.15	6.00	7.00	8.00
4	5.3	33.5	49.8	37.8	24.0	7.0
8	9.5	52.3	59.7	44.0	36.3	19.5
12	10	55.8	61.5	48.7	42.0	22.5
24	11.5	63.7	71.5	53.3	47.3	24.5

TABLE 3.7 The effect of pH (McIlvaine's Buffer) on urediospore germ-tube length after 30 h.

Average germ-tube lengths in µm	pH					
	3.10	4.10	5.15	6.00	7.00	8.00
	56.3	121.9	140.8	86.7	53.2	39.8

Fig. 3.6. The effect of pH (McIlvaines Buffer) on U. dianthi urediospore germination (●—●) and germ-tube length (o- -o).

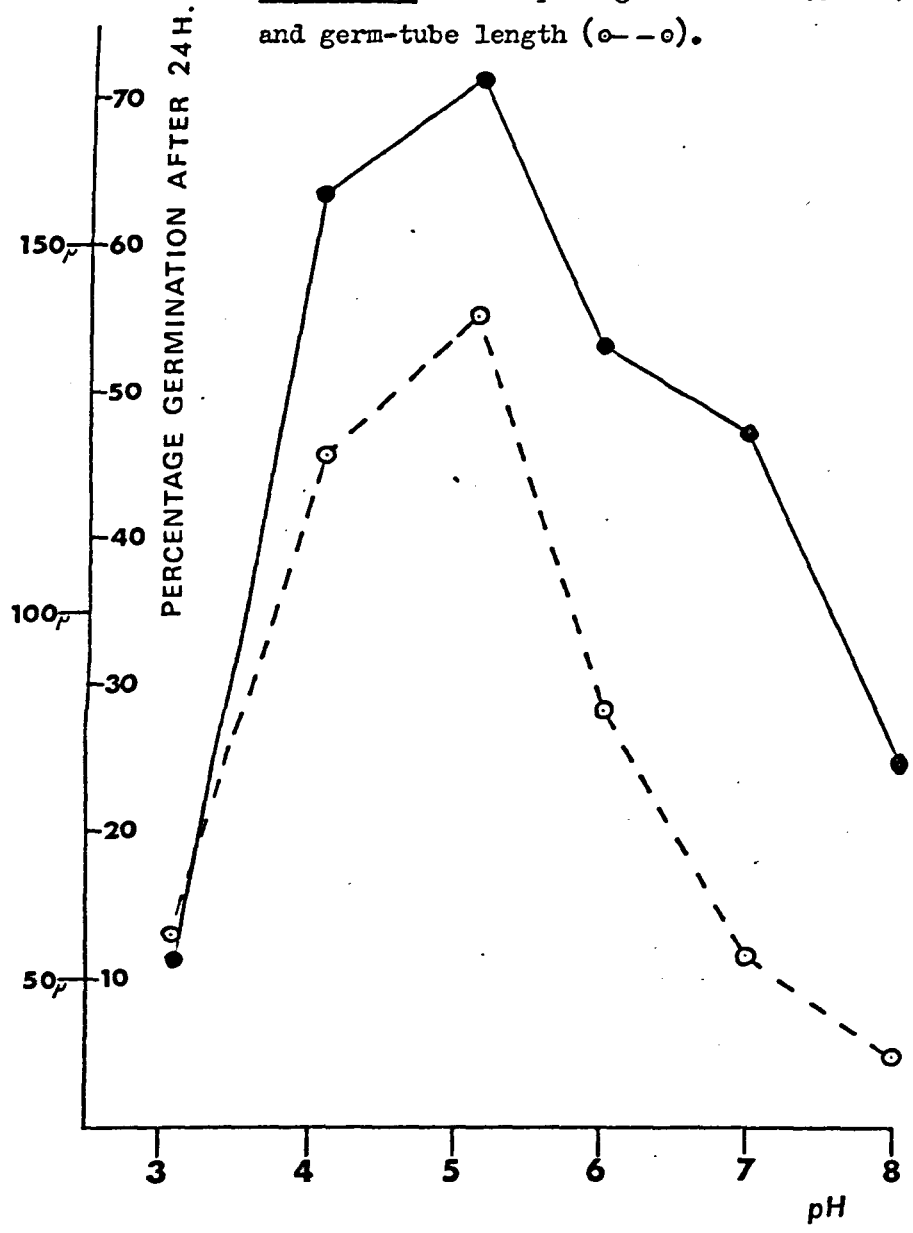
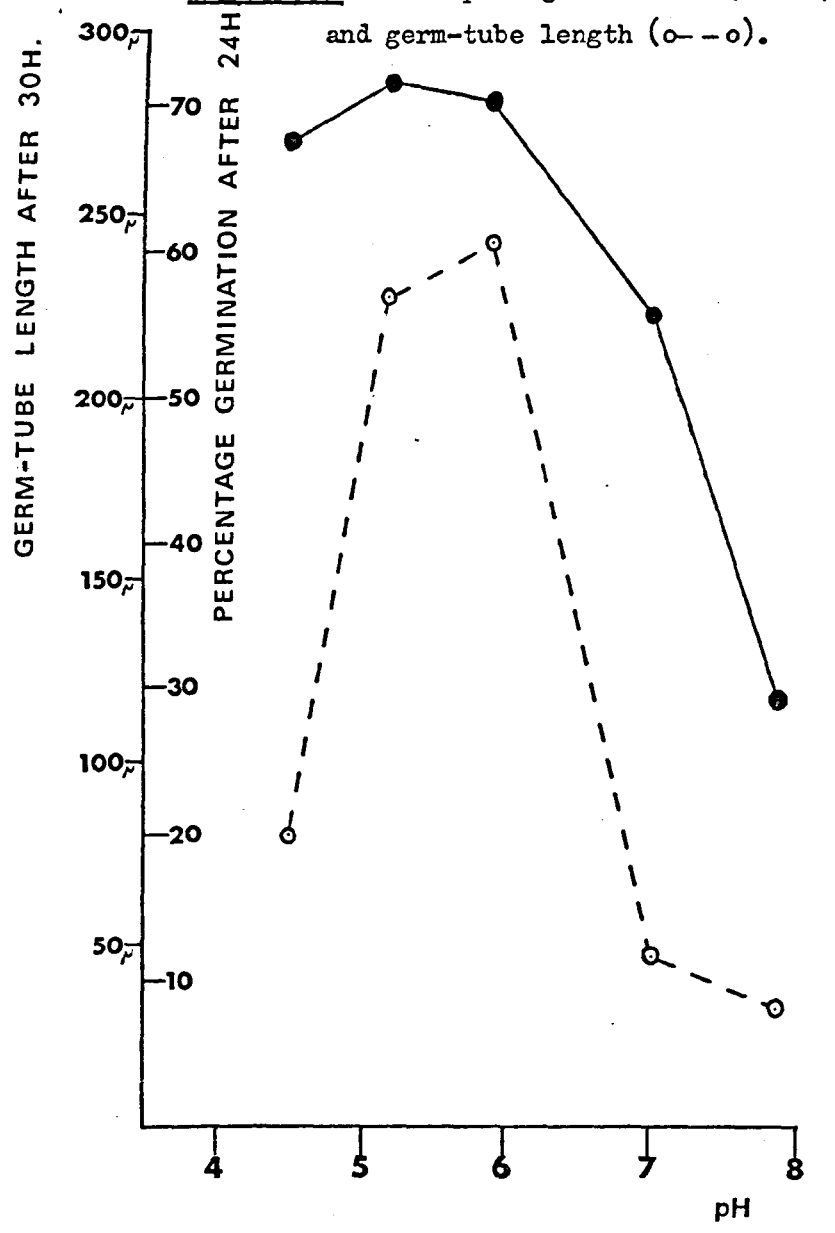


Fig. 3.7. The effect of pH (Sorensens Buffer) on U. dianthi urediospore germination (●—●) and germ-tube length (o- -o).



The physical state of the germ-tubes varied as follows:

pH 3.1 - Stunted germ-tubes

pH 4.1 - Corkscrew shaped germ-tubes

pH 5.15 - Normal, slightly coiled germ-tubes

pH 6.0 - Straight germ-tubes

pH 7.0 - Short straight germ-tubes

pH 8.0 - Very short germ-tubes. Growth of the tube stops soon after emergence.

With Sörensen's Buffer, a germination optimum of around pH 5.2 was indicated. (Table 3.8 and 3.9, Fig. 3.7). No statistically significant difference was found however between the germination readings taken at 8, 12, and 24 h on the buffer solutions of pH 4.5, 5.2 and 5.9.

The results from both buffer systems indicate a germination optimum of approximately pH 5.2

3.33 U. dianthi urediospore exudate studies.

3.33.1 The effect of a urediospore exudate solution on urediospore germination.

Experimental procedure. Exactly 5 mg of urediospores were weighed into a small clean sterile staining block and then 1 ml of 0.1 M sterile Sörensen's buffer of pH 5.5 (approximate optimum germination pH for buffer) was run into the block from a sterile pipette. Buffer was used in place of water in order to cushion pH changes due to the diffusion of organic acids, etc. from the urediospores. The spores were evenly distributed over the surface of the buffer by agitation with sterile needles. The staining block cover plate was then sealed on with petroleum jelly and the block was incubated for 10 h at 20°C. It was considered that after 10 h any exudate would have diffused out into the buffer. After 10 h, the percentage germination

TABLE 3.8 The effect of pH (Sörensen's Buffer) on urediospore germination.

Time in h	Germination Percentages				
	pH				
	4.50	5.20	5.90	7.00	7.85
4	55.7	64.5	55.8	38.3	14.7
8	63.0	68.7	64.2	50.8	21.0
12	64.8	69.3	66.7	53.0	28.7
24	67.5	71.6	70.2	55.5	29.2

TABLE 3.9 The effect of pH (Sörensen's Buffer) on urediospore germ-tube length after 30 h.

Average germ-tube lenghts in µm	pH				
	4.50	5.20	5.90	7.00	7.85
	80.8	227.2	242.8	46.7	32.4

of the spores was below 1%.

The spore exudate solution was removed from the block by means of a Pasteur pipette. Few spores were removed from the block as the end of the pipette was placed into the buffer solution below the floating spore layer. Fresh sterile buffer (1 ml) was run into the staining block which was incubated for 4 h at 20°C. After this time an estimated 58.8% of the spores had germinated indicating that most of the inhibiting exudate present had been removed with the initial 1 ml of buffer, and that a considerable proportion of the inhibitor had diffused from the spores in the 10 h.

The original 1 ml of buffer (spore exudate) was used in hanging drop germination experiments. Freshly collected urediospores were mixed into the spore exudate solution in a staining block and five drops taken with a sterile eye lotion dropper. As a control, fresh sterile buffer was used to make the solution of similar spore concentration. Five drops were removed by a sterile eye lotion dropper for control hanging drop germination tests.

In each drop germination was assessed from 200 spore observations after 4, 8, 12 and 24 h. The final germination percentage for the buffer control and the spore exudate drops were therefore calculated from 1000 spore observations each.

Results. The urediospore exudate solution had an inhibitory action on urediospore germination (Table 3.10, Fig. 3.8). The inhibitory action of the exudate solution decreased slowly with time. This could be explained by partial volatilisation of the inhibitor.

TABLE 3.10. The effect of urediospore exudates on urediospore germination.

Time in h	Germination Percentages	
	Buffer control	Buffer Exudate
4	49.6	8.0
8	62.3	12.5
12	66.4	20.7
24	69.1	25.7

TABLE 3.11 The effect of dilution of urediospore exudate solutions on urediospore germination.

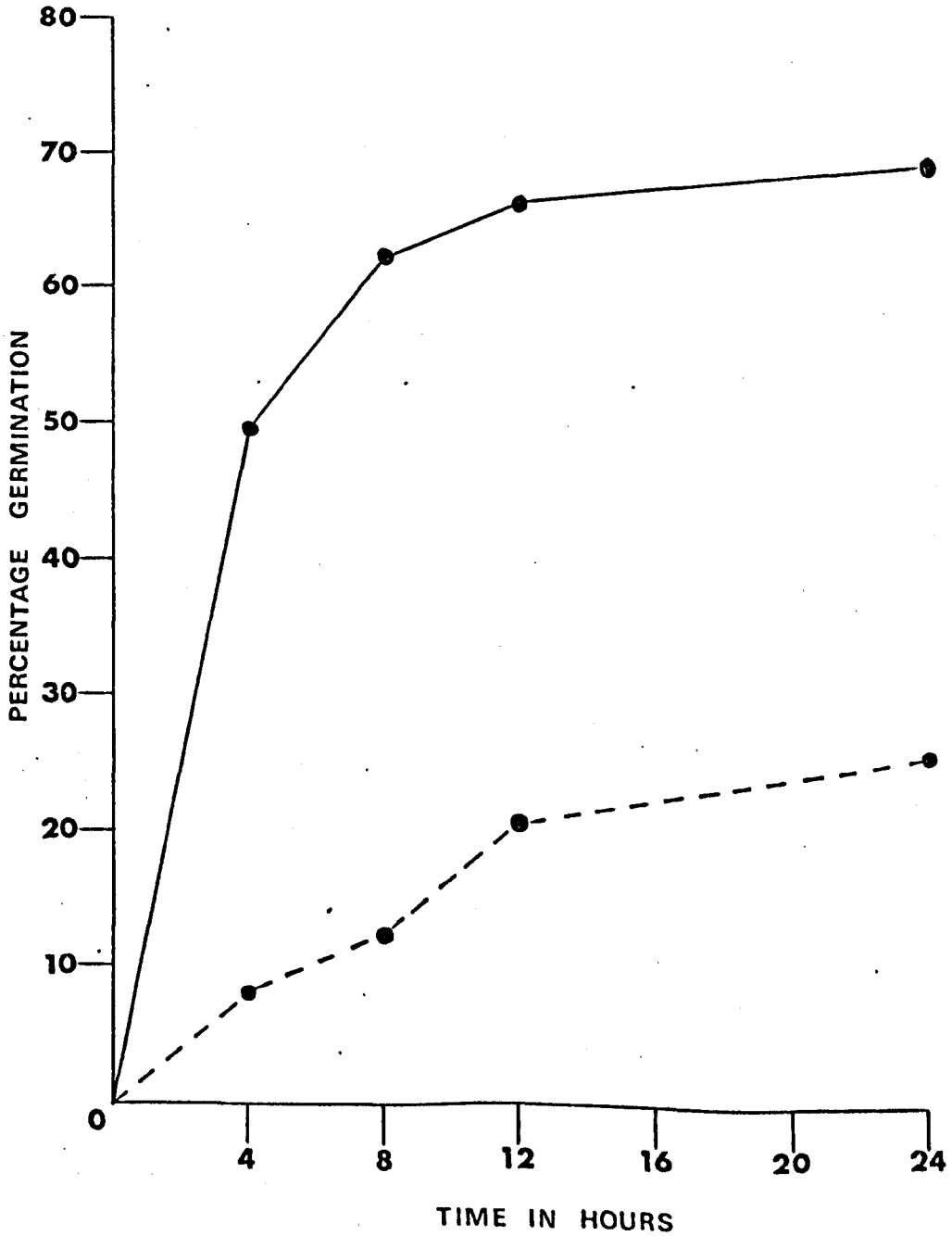
Percentage Concentration of Exudate	% Germination After 10 h	
	Sörensen's Buffer	Distilled Water
0	38.2	92.6
12.5	24.2	84.8
25	19.8	79.0
50	8.7	68.6
75	3.8	58.8
100	1.1	39.6

TABLE 3.12 The effect of dilution of urediospore exudate solutions on germ-tube length. (Sörensen's Buffer Expt.).

% of Exudate	0	12.5	25	50	75	100
Germ-Tube length	168.7 µm	-	171.7 µm	196.0 µm	202.0 µm	251.9 µm*

*Average of 40 germ-tube measurements

Fig. 3.8. The effect of a U. dianthi urediospore exudate solution on U. dianthi urediospore germination (●———● control, ●– – –● exudate).



3.33.2 The effect of dilution on the inhibitory action of urediospore exudate solutions.

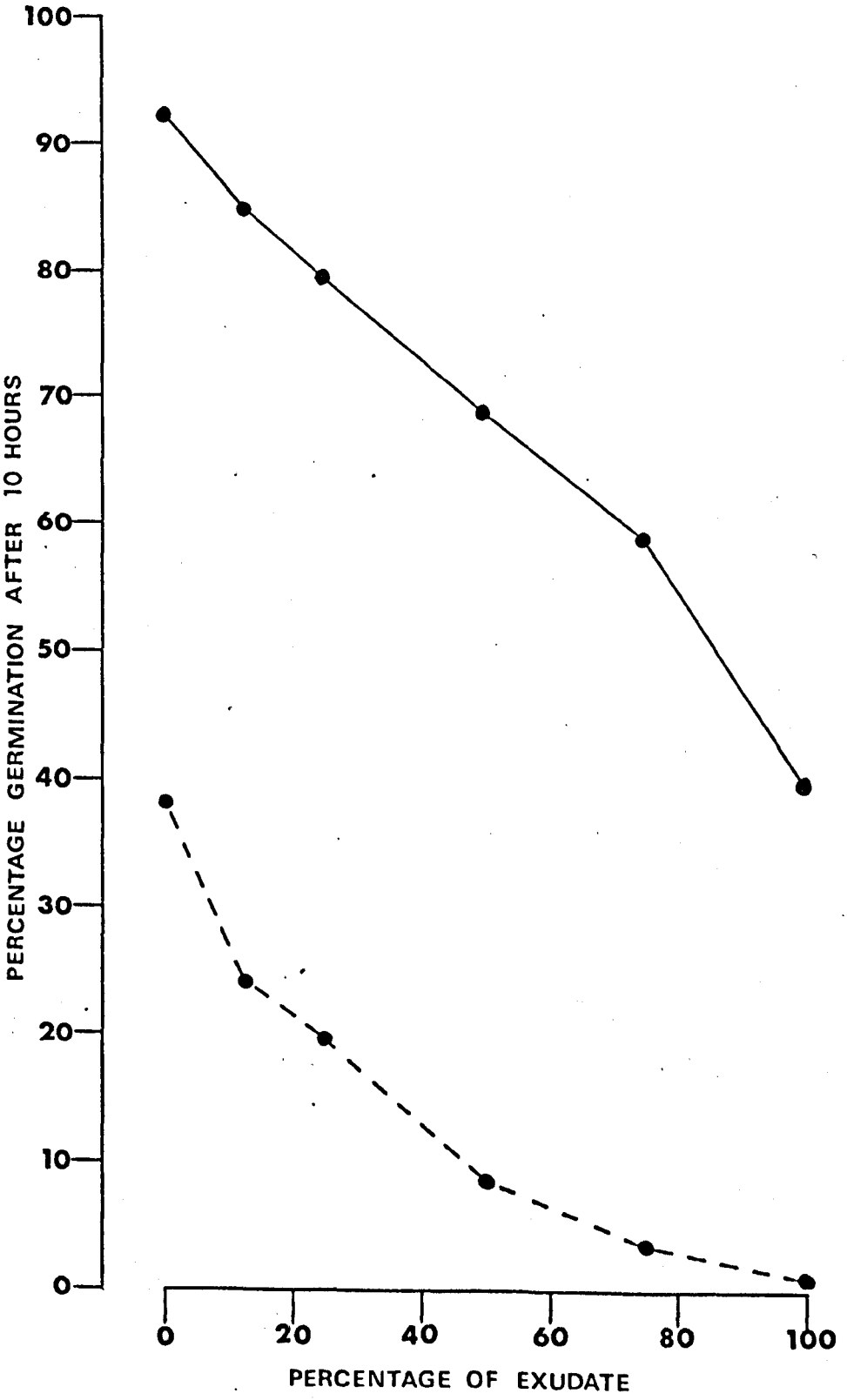
Experimental procedure. In order to obtain an exudate solution, 25 mg of urediospores were weighed, placed in a Warburg flask and 5 ml of 0.1 M Sörensen's Buffer (pH 5.5) added. The flask was sealed and incubated for 12 h at 15°C. After 12 h, the exudate solution was removed by a Pasteur pipette. The solution was placed in a test tube and dilutions made using Sörensen's Buffer. Two 0.4 ml samples of each dilution were pipetted into staining blocks. The dilutions contained 75%, 50%, 25% and 12.5% of the original exudate. Two 0.4 ml portions of the original exudate (100%) and pure Sörensen's buffer (0%) were also used.

A number of urediospores (approximately 1000) were then transferred to the surface of the solutions using a camel hair brush. The cover plates of the staining blocks were sealed with petroleum jelly, and the spores incubated for 10 h at 20°C. After this time, 500 spores observed in each staining block were used to assess germination. The germination percentage for each dilution was therefore calculated from 1000 urediospore observations. After 11 h at 20°C, average germ-tube lengths were estimated by the measurement of 50 germ-tubes in each dilution.

The experiment was repeated using sterile distilled water (pH 5.3) in place of Sörensen's Buffer. Only one staining block containing a dilution of exudate solution was used and the germination percentages were calculated from 500 urediospore observations.

Results. As the concentration of urediospore exudate in the solutions increased, the germination percentage of the urediospores decreased (Table 3.11, Fig. 3.9).

Fig. 3.9. The effect of dilution of U. dianthi urediospore exudate solutions on U. dianthi urediospore germination (●———● exudate in distilled water, ●- - -● exudate in Sorensen's Buffer).



The differences in total urediospore germination percentages found between dilution in Sörensen's Buffer (low germination) and distilled water (high germination) can be explained by the age of the urediospores. In the initial buffer experiment the urediospores used for the germination tests were from old urediosori and contained a high proportion of physiologically inactive spores. In the distilled water experiment, younger urediospores were used.

The germ-tubes of the urediospores that germinated in the high concentrations of exudate were on the average longer than those in the low concentrations (Table 3.12).

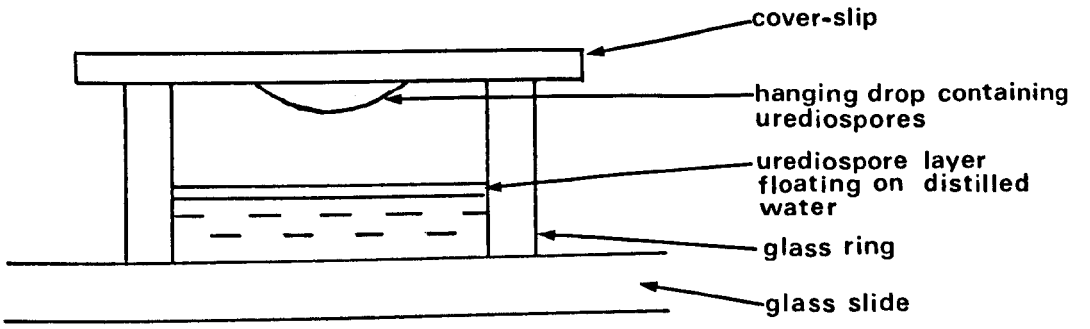
3.33.3 The effect of the urediospore exudate solution on urediospore germination across an air gap.

Experimental procedure. In this experiment, a modification of the hanging drop technique was utilised. Urediospores (0.2 mg) were weighed into a well formed by a glass ring attached by Neutral Canada Balsam to a glass slide, and then 0.4 ml of sterile distilled water was pipetted into the well. A cover slip with a hanging drop containing urediospores in sterile distilled water, was then sealed on the glass rings with petroleum jelly (Fig. 3.10).

A control drop was sealed over a glass ring in which the well contained 0.4 ml of sterile distilled water but no urediospores. The experiment had two replicates. All the hanging drops were derived from a spore solution in a small staining block, and were approximately equal in size, containing about 1000 spores.

Germination readings of the spores in the drops were taken after 2, 4, 8 and 12 h at 20°C. In each drop 200 spores were observed and total germination was therefore calculated from

Fig. 3.10. Modified hanging drop technique to find the effect of U. dianthi urediospore exudate solutions on the germination of U. dianthi urediospores across an air gap.



600 observations. After 11 h the average germ-tube length was estimated from the measurements of 100 germ-tubes.

The whole experiment was repeated using 0.2 ml of sterile distilled water in the glass ring wells, and omitting the measurement of the germ-tube lengths.

Results. A significant though weak inhibitory action on germination occured in the hanging drops, thus the exudate inhibitor in the well was active across an air gap. (Table 3.13, Figs. 3.11 and 3.12).

There appeared to be no significant difference in the length of germ-tubes in the control and exudate solution's hanging drops (Table 3.14).

3.33.4 The effect of distillation on the inhibitory action of a urediospore exudate solution.

Experimental procedure. Urediospores (20 mg) were weighed out in a Conway micro-diffusion dish, and then 5 ml of sterile double distilled water was pipetted in and the dish sealed. The spores formed a continuous layer on the water surface.

TABLE 3.13 The effect of urediospore exudate solution on urediospore germination across an air gap.

Time in h	Germination Percentages			
	0.4 ml of d.w. in Wells		0.2 ml of d.w. in Wells	
	Control	Exudate	Control	Exudate
2	38.3	22.2	42.8	28.5
4	50.2	38.7	60.8	42.0
8	58.3	46.0	68.3	46.5
12	59.8	48.5	71.2	50.8

TABLE 3.14 The effect of urediospore exudate solution on urediospore germ-tube length across an air gap.

Average Lengths	
Hanging Drop - Control	Hanging Drop - Exudate
277.2 µm	273.8 µm

Figs. 3.11, 3.12. The effect of U. dianthi urediospore exudate solutions on U. dianthi urediospore germination across an air gap (●——● control, o- - - -o exudate).

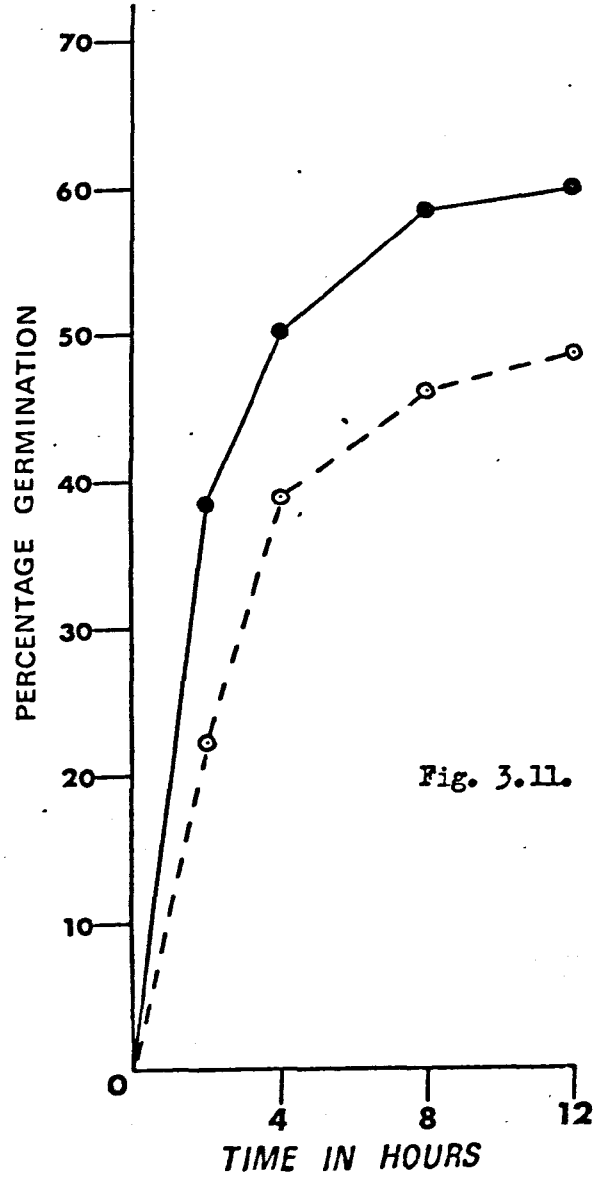


Fig. 3.11. 0.4 ml of distilled water in wells with urediospores.

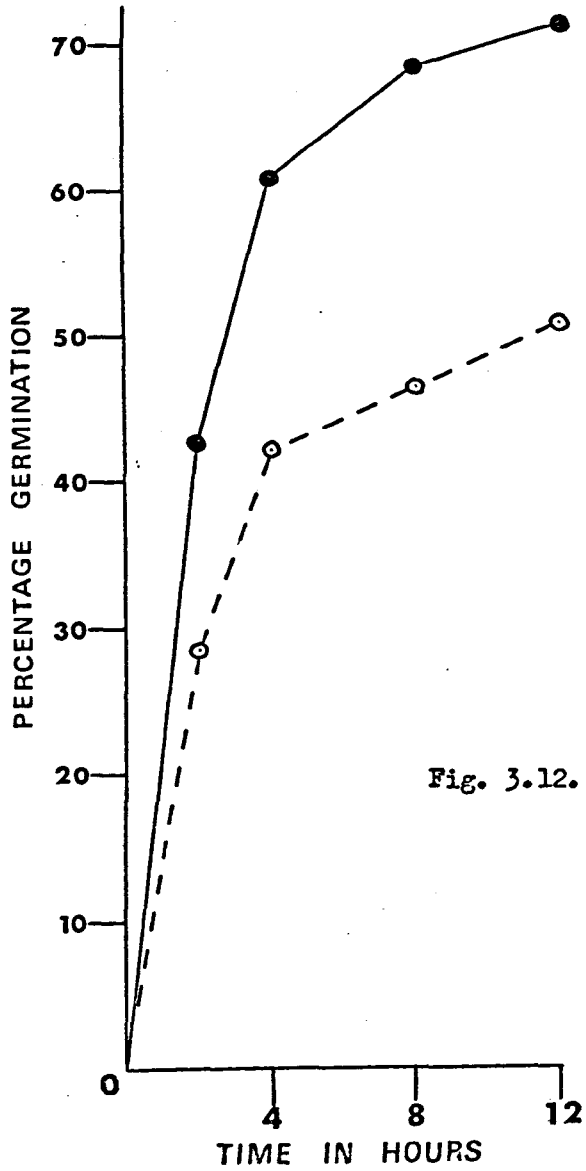


Fig. 3.12. 0.2 ml of distilled water in wells with urediospores.

The dish was incubated for 12 h at 15°C. After incubation no spores were observed to have germinated. The exudate solution was drawn off by Pasteur pipette and ejected into a glass tube. A spore free solution was obtained. Four drops were placed on clean cover slips and urediospores sprinkled on from a small camel hair bursh. Four control hanging drops were prepared with sterile double distilled water. Approximately equal amounts of urediospores were added to each of the drops, which were incubated at 20°C. Germination readings were taken after 2 and 4 h.

The remainder of the exudate solution was placed in a micro-distillation apparatus. The solution was heated to boiling with a micro-Bunsen and the distillate collected in a small flask in an ice bath. Some of the distillate was used in four hanging drops which were incubated at 20°C and germination readings taken after 2 and 4 h. Two hundred spores were observed for germination in each drop. The results from the four drops were averaged to give a percentage.

Result. There was no significant statistical difference between the germination readings before and after distillation (Table 3.15). The inhibitor therefore must be thermostable.

3.33.5 To find which fraction of a distillate of a urediospore exudate solution has inhibitor activity.

Experimental procedure. Urediospores (20 mg) were weighed out in a Conway micro-diffusion dish, 5 ml of sterile double distilled water added and the plate glass lid sealed. The spores were incubated floating on the water surface of 12 h at 15°C. The exudate solution was removed by Pasteur pipette to a graduated plastic centrifuge tube. Some of the exudate solution (1.0 ml) was distributed between three staining blocks

TABLE 3.15 The effect of distillation on the inhibitory action of a urediospore exudate solution.

Time in h	Germination Percentages		
	Inhibitor before Distillation	Inhibitor after Distillation	Control (Distilled Water)
2	21.5	19.4	54.4
4	38.3	35.8	79.5

TABLE 3.16 Fractions of a distillate of a urediospore exudate solution and their effect on urediospore germination.

Time in h	Germination Percentages					
	Control	Exudate (Before distil-lation)	Distillates			
			1 100-105°C	2 105-115°C	3 115-140°C	4 140-160°C
2	58.8	15.0	41.3	30.3	20.8	4.8
4	81.3	36.0	63.8	54.2	40.5	11.3

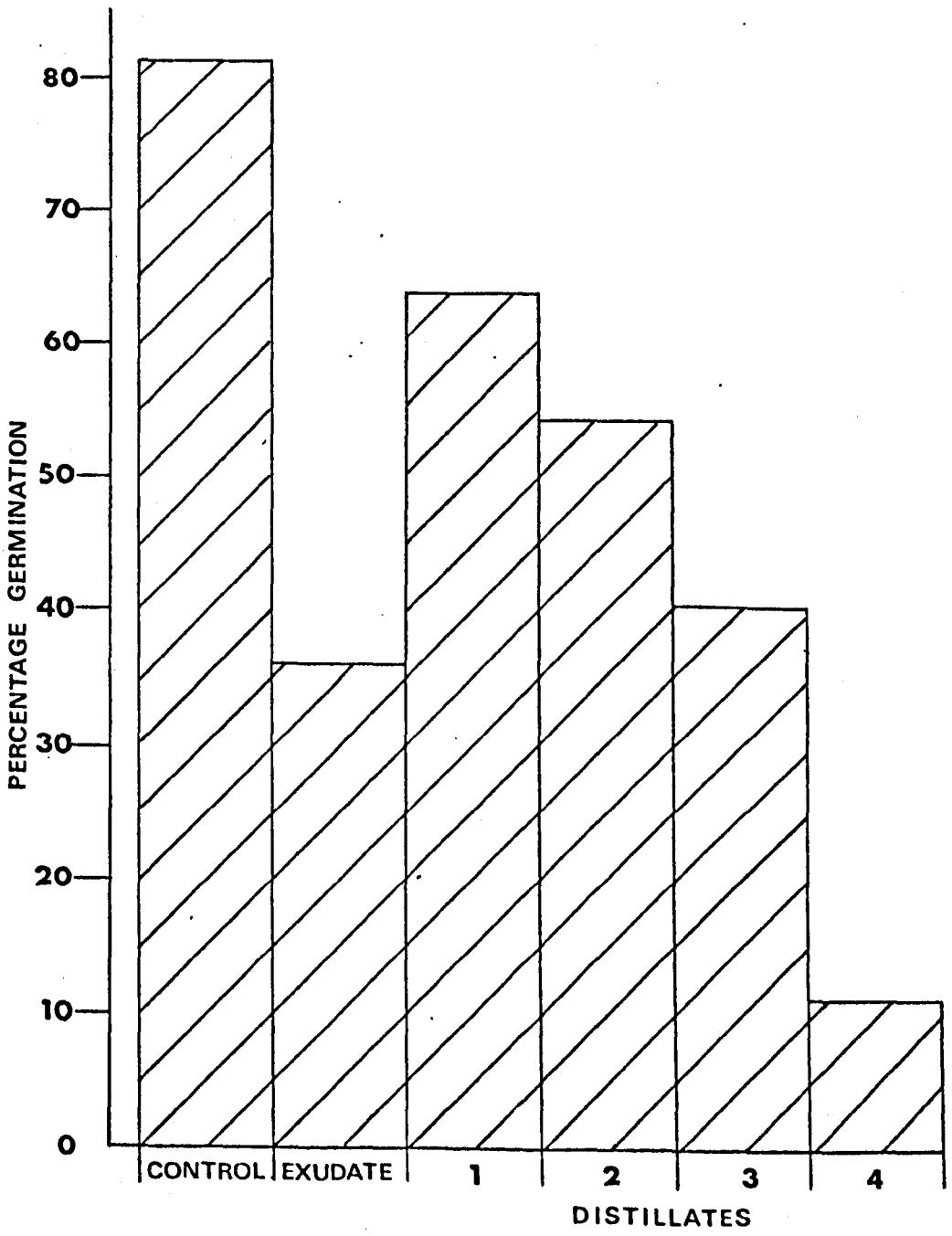
and surface sprinkled with approximately equal numbers of urediospores. The blocks were sealed with glass covers and petroleum jelly. Control staining blocks were prepared in the same way with double distilled water. The remaining 4 ml of inhibitor was poured into a micro-distillation apparatus. The distillate was collected in plastic graduated centrifuge tubes. The tubes were changed after each 1 ml of distillate had come over. The tubes were numbered 1 to 4, the distillate in tube 1 coming over between 100-105°C, the distillate in tube 2 between 105-115°C, the distillate in tube 3 between 115-140°C and the distillate in tube 4 between 140-160°C. Each ml of distillate was distributed between three staining blocks and equal amounts of urediospores sprinkled on the solution surface. Germination readings were taken after 2 and 4 h at 20°C. In each staining block 200 spores were observed for germination. The results for each block were averaged as a percentage.

Results. The last fraction of the distillate appeared to have the greatest inhibitor activity (Table 3.16, Fig. 3.13).

3.4 DISCUSSION

The optimum temperatures for U. dianthi urediospores germination as found by Doran (1919) and Székely (1967) were 14°C and 24°C respectively. In the present experiments (see 3.42), the optimum germination temperature was between 20°C and 22.5°C. If these workers used water drops in their germination experiments containing large number of urediospores, the differing results could be explained by the effects of germination inhibitors. Another possibility is that different physiological races of U. dianthi have evolved different optimum germination conditions. Although the minimum temperature of urediospore

Fig. 3.13. A comparison of the effects of a U. dianthi urediospore exudate solution and fractions of its distillate on U. dianthi urediospore germination after 4h at 20°C.



germination recorded by Doran (1919) was 4°C and by Székely (1967) 2°C, U. dianthi urediospores were found to germinate at 1°C in the work being described (Table 3.2). Good urediospore germination was recorded at 30°C (Table 3.2), although Doran (1919) found 29°C to be the maximum temperature at which germination was possible. This also could be explained by an intereaction between inhibitors in water drops containing large numbers of urediospores, and temperature.

The criteria used by these authors to measure germination are not stated. It is possible that Doran (1919) considered that a urediospore had germinated when its germ-tube was half the minor diameter of the spore (American Phytopathological Society, 1943), or even when the tube was greater than the diameter of the spore. (French et al., 1957). This of course would explain the maximum and minimum temperatures of 29°C and 4°C as U. dianthi urediospore germ-tubes rarely developed at those temperatures. It is therefore important that authors state their germination criteria.

It is also considered important that the rate of urediospore germination is known, as this is often more sensitive to temperature, than is the total number of spores which eventually germinate. Doran (1919) assessed germination 'within 24 h, usually within 12 h'.

It has been found that the optimum temperature for germination of spores need not necessarily be the same as the optimum for germ-tube growth. The results indicated that with U. dianthi however, the two temperatures were very similar, if not identical.

The pH germination experiments found that pH 5.2 was approximately the optimum pH for germination. This is similar to the pH of distilled water, rain water, and dew (pH 5.2 - 5.3). In natural conditions therefore, it seems likely that spores

would germinate on the leaf surface in water of the optimum pH value. Exudates from the leaf surface might not appreciably alter the pH in the short time needed for germination and infection.

The behaviour of the germination inhibitor present in U. dianthi urediospore exudate solutions in some ways resembled the inhibitor found in exudates of P. graminis f. sp. tritici urediospores (Allen, 1955). These were:- (1) spores floated en masse for 10 - 12 h were so altered that they no longer showed any signs of self inhibition when placed on a fresh solution en masse. (2) The solution on which urediospores had been floated for 10 - 12 h contained a substance or substances active in reducing germination of other urediospores. (3) An actively metabolising population of spores brought about some inhibition in other spores separated from them by an air gap.

Allen (1957) found that the exudate solution from P. graminis f. sp. tritici urediospores was clear and light yellow in colour, and that when distilled a germination stimulant was formed. Most of the stimulatory activity was in the first 10 to 20% distilling over. This is in contrast with the clear colourless exudate solution of U. dianthi urediospores which retained its inhibitory activity after distillation, most of the inhibitor coming over in the last 25% fraction. The inhibitory action of U. dianthi urediospores also seemed to be very much less than that of P. graminis f. sp. tritici urediospores. When the P. graminis inhibitory solution was diluted, the dilution activity curves obtained showed that considerable inhibition still occurred at a 10 x dilution, and at 2 x dilution the inhibition was almost as great as the crude exudate. With the U. dianthi urediospores the inhibitory activity diminished considerably on dilution (Fig. 3.7). These results may be

may be related to a loss of activity due to contact with glass surfaces (Allen, 1955).

Forsyth (1955) absorbed the volatile P. graminis f. sp. tritici inhibitor into acetone and found that the absorption spectra of the inhibitor was similar to that of trimethylethylene. His experiment was repeated using U. dianthi urediospores, but nothing was found in the acetone.

It seems therefore that U. dianthi urediospores exude in solution a partially volatile, thermostable germination inhibitor, which has properties different from those found in P. graminis f. sp. tritici urediospores. It is possible that a number of inhibitors are produced which have different chemical and physical properties. This could explain the fact that the inhibitor is volatile and also comes over in the last fraction when distilled.

Simultaneous self-stimulation and self-inhibition of the germination of U. phaseoli urediospores has been reported by Yarwood (1956). As germination decreased due to denser concentrations of urediospores, the average germ-tube lengths were found to increase. The dilution of a U. dianthi crude spore exudate solution also reduced the average length of growing germ-tubes (Table 3.12). It could be argued that a germ-tube stimulator present in high concentrations in the crude exudate solution was also diluted. It is possible therefore that a self-stimulator is released by U. dianthi urediospores. The nature of the self-stimulator is unkown, but it may be a combination of the effects of 'leaking' urediospore metabolites such as amino acids, carbohydrates, etc. It does not seem to be volatile as there appears to be little or no effect on germ-tube length across an air gap. (Table 3.14).

CHAPTER 4. AXENIC CULTURE INITIATION

4.1 INTRODUCTION

Axenic culture is defined as the growth of a single species in the absence of living organisms or living cells of any other species (Dougherty, 1953). That is, the growth of an organism on non-living substrata.

The first successful *in vitro* culture of a rust was achieved by Hotson & Cutter (1951). *Gymnosporangium juniperi-virginianae* was cultured from systemically infected callus tissue cultures of *Juniperus virginiana* telial galls. In one four month old callus culture the rust grew out from the tissue and invaded the nutrient medium. The mycelium when transferred directly to fresh nutrient continued to grow. The medium used was Gautheret's nutrient No.4 solution modified by 3% dextrose, 500 p.p.m. ascorbic acid, 1% yeast extract, and 10^{-7} g/l naphthalene acetic acid.

Cutter (1960a) also cultured *Uromyces ari-triphylli* from systemically infected corms of *Arisaema triphyllum*. As he described for *G. juniperi-virginianae*, a necrotic reaction was observed in the host tissues prior to the emergence of all saprophytic strains of *U. ari-triphylli*. Cutter (1959) suggested that this reaction in the host callus selected for variant nuclei with saprophytic tendencies.

The obvious starting material for the majority of workers attempting axenic cultures have been spores, as these are produced by the rust for its natural dissemination. Characteristic spores are formed at different stages of a rust's life cycle (Webster, 1970). Most work has been carried out using urediospores, as they are the most abundant, uniformly produced, readily obtainable spores. Early workers had little success (Brefeld, 1883; Mains, 1917) due to contamination, faulty

technique, or unsuitable media. This led to an assumption by many that the axenic culture of rusts and other obligate fungal plant parasites was impossible.

In recent years however three further rust fungi have been successfully grown from uncontaminated urediospores in axenic culture. Williams, Scott & Kuhl (1966) and Williams, Scott, Kuhl & Maclean (1967) found that an agar medium consisting of Czapek Dox salts, sucrose, Difco Yeast Extract, and Evans's Peptone not only initiated and supported the saprophytic growth of race ANZ - 126 - 6 of P. graminis f. sp. tritici from germinating urediospores, but also induced the fungal colony to sporulate. Other workers have successfully cultured Puccinia recondita f. sp. tritici (Singleton & Young, 1968) and Melampsori lini (Turel, 1969; Coffey, Bose & Shaw, 1970) on similar media. Gelatin suspensions of P. graminis f. sp. tritici urediospores were found by Coffey, Bose & Shaw (1969) to initiate better growing axenic cultures, than urediospores suspended in water. Bushnell & Rajendren (1970) found media supplemented with casein hydrolysate useful when culturing P. graminis f. sp. tritici. Such results suggested that the problem of culturing rusts was primarily one of nutrition.

Since 1970, workers have concentrated on understanding the processes leading to initiation of axenic cultures from P. graminis f. sp. tritici urediospores, and on the physiology and genetics of the saprophytic rust mycelium.

Kuhl, Maclean, Scott & Williams (1970) found that the frequencies of growth initiation and colony formation of P. graminis f. sp. tritici race 126 - ANZ - 6, 7, were highest on the thinnest layers of nutrient agar and that sporelings mutually stimulated each other.

Two distinct categories of axenic culture growths of P.

graminis f. sp. tritici race 126 - ANZ - 6, 7, were recognized by Maclean & Scott (1970). The more common type formed macroscopic colonies of binucleate cells within 2 weeks of inoculation. Growth ultimately ceased as a result of staling and the formation of a spore-bearing stroma. The other category was only occasionally formed, had uninucleate cells, and showed a much less tendency to stale or form stromata and could be subcultured.

Bushnell & Stewart (1971) carried out axenic culture initiation experiments with twenty-five deverse American isolates of P. graminis f. sp. tritici with varying degrees of success.

Working with P. graminis f.sp. tritici race 126 ANZ - 6, 7 Williams (1971) found that dikaryotic mycelia were formed only by urediospores which differentiated an infection structure, but monokaryotic mycelia arose from germ-tubes which failed to differentiate.

Contrary to this, Bose & Shaw (1971) working with the same race of P. graminis f. sp. tritici stated that dikaryotic mycelia developed from centres of germ-tube anastomosis without the formation of typical infection structures.

Diploid lines of P. graminis f. sp. tritici have been found in axenic culture. This showed that diploidization can take place in vegetative mycelium (Hartley & Williams, 1971a). Further investigations suggested to Hartley & Williams (1971b) that genotypic variation within a phenotype was a possible basis for somatic hybridization in rust fungi.

Hartley & Williams (1971c) have also carried out work on interactions between strains of P. graminis f. sp. tritici in axenic culture. An isolate with the virulence of one strain but having improved capabilities as a saprophyte was recovered from a mixed culture. They also found morphological and

cultural differences between races (Hartley & Williams, 1971d).

Attempts to culture rusts from other spore types have generally proved unsuccessful. However, Bailey (1923) mentioned frequent "indefinite growth" and branching of promycelia from germinating Puccinia helianthi teliospores, and Reed & Crabhill (1915) described the formation of a narrow twisted hypha without basidiospore formation when teliospores of Cronartium ribicola were germinated under water.

The first axenic culture of a rust from spores may have in fact been accomplished by Cutter (1960b). Three month old Althaea callus tissues infected with the microcylic rust Puccinia malvacearum developed telial pustules bearing mature teliospores. In one culture a minute fungal colony was noted on the agar surface about 8 mm from the callus. Cutter thought that the colony may have arisen from the germination of a basidiospore. Later attempts to obtain axenic growths by seeding basidiospores on nutrient agar were however unsuccessful.

In the work to be described, attempts were made to initiate axenic cultures of U. dianthi from urediospores, and P. arenariae from basidiospores.

4.2 MATERIALS AND METHODS

4.21 U. dianthi axenic culture experimental methods.

4.21.1 Techniques for obtaining aseptic urediospores.

By using techniques similar to those of Williams et al. (1966) large numbers of uncontaminated urediospores were obtained. The upper surface of young uninfected detached carnation leaves (var. Grenadin Scarlet) was sprayed with atomised water and dusted with urediospores. With their adaxial surfaces uppermost the leaves were then floated on water in Petri dishes sealed with adhesive tape. After incubation for 48 h

at 20°C the Petri dishes were moved to a growth cabinet (12 h day, 18 - 22°C). Two or three weeks after inoculation the characteristic early flecking symptoms became visible. The infected leaves were then surface sterilised by a 10 min immersion in a solution of 5% sodium hypochlorite plus a few drops of Tween 80. This sterilization procedure was found to kill most of the phylloplane bacteria, the main source of contamination, and yet enable the sori to develop and produce urediospores. After washing for 10 min in three changes of sterile distilled water, the leaves were placed on the surface of Oxoid Nutrient Agar in Petri dishes. The Petri dishes were then stacked in a desiccator and returned to the growth cabinet. The aseptic urediospores became exposed as the sori ruptured the epidermis, and were placed directly on the surface of the various agar media with either sterile inoculating needles or sterile cotton buds.

In later initiation experiments, urediospores were taken directly from growing plants. The spores were removed with a sterile scalpel blade from young opened sori and quickly transferred to the agar medium. In one experiment 20% of the total transfers were contaminated. However, the percentage germination of these urediospores was noted to be higher, subsequent germ-tube growth more profuse, and saprophytic initiation more common, when compared with the behaviour of spores from leaves subjected to the surface sterilization procedure.

4.21.2 Subculturing of U. dianthi in axenic culture.

The growing margins of colonies were broken up into small pieces, usually 1 - 3 mm^2 with sterile scalpel blades, which were then used to transfer the fragments of colony to the surface of the new medium. Occasionally a colony would detach itself from the agar while being broken up. When this

happened, the colony was transferred to a sterile empty plastic Petri dish and the margins quickly fragmented. The fragments were then speedily transferred to the fresh medium before the death of the mycelium due to desiccation.

Sharp scalpel blades were used throughout the subculturing procedure, as they enabled the colony edge to be cut more easily. Before use, the blade was flamed to red heat, and before and after each subculture transfer, the blade was dipped in alcohol and flamed. Prior to cutting and transferring, the warm blade was cooled by submergence in sterile agar.

All subculturing was carried out at room temperature in sterile inoculating rooms. Petri dishes containing subcultures were sealed with adhesive tape to reduce the risk of contamination and to slow the dehydration of the medium caused by evaporation. Usually between 35 - 40 ml of agar medium were poured into each Petri dish. Plates were incubated in the normal position as inverted subcultures often became detached and fell on the petri dish lid.

4.21.3 Measurements of U. dianthi colony diameters.

On Petri dishes, the linear growth in mm was measured as the colony diameter. Where the colony was not circular in shape, the long and the short axes were measured and the average taken as a measure of growth.

4.22 P. arenariae axenic culture attempt experimental methods.

4.22.1 Techniques used for obtaining aseptic basidiospores.

Sweet William infected with P. arenariae were obtained from a grower near Biggleswade, Bedfordshire. These were used to infect young Dianthus barbatus var. Scarlet Beauty. Leaves with young opened teliosori containing ungerminated teliospores were detached from the plant and the teliosori isolated on small lengths of cut leaf. The surface of the leaf was then sterilized with a 10% solution of sodium hypochlorite applied

with a cotton bud. The leaves, taking care to leave the teliosori exposed, were then carefully attached to the inside of the centre of Petri dish lids by means of waterproof adhesive tape. The Petri dish lids were then quickly placed over plates of culture media. The plates were placed in 20°C incubators (optimum germination temperature for P. arenariae teliospores, see Chapter 3.41.1).

The humid atmosphere in the plates led to the germination of the teliospores and the discharge of basidiospores over the agar surface. After discharge (6 - 18 h), the Petri dish lids with the attached leaves with teliosori, were removed to prevent contamination. These were replaced with new sterile lids and sealed with adhesive tape. Despite the caution, between 20 - 30% of the plates became contaminated with the yeast Sporobolomyces roseus. The plates were incubated at 18 and 20°C in the dark.

4.23 Preparation of axenic culture media.

All culture media constituents were weighed accurately on Oertling R20 balances. Agars, Difco Czapek Dox Broth, peptones, yeast extracts, and casein hydrolysates, were usually weighed on Whatman filter papers and tipped into beakers. Occasionally, when extreme accuracy was needed,watch glasses were used for weighing, and the constituents washed off with distilled water. Known volumes of water were added to the beakers and the pH of the resulting solutions adjusted using 0.1 N and 0.01 N-HCl. Usually the pH was altered to approximately 0.1 pH below that which was desired, as on autoclaving the pH rose to the required value. pH readings were taken in the laboratory using a portable glass electrode pH meter (Pye model 79). The pH meter was standardised against a buffer solution before and after each measurement.

Agars were usually weighed directly into the autoclaving flasks as they did not greatly effect the pH of the medium. Czepek Dox Agar however was added to the constituent solution in the beaker. The solutions were poured through glass funnels into the autoclaving flasks. Generally media were sterilized by autoclaving at 120°C for 15 min.

4.3 RESULTS.

4.31 In vitro culture of U. dianthi

4.31.1 First initiation of an axenic culture and observations.

The first successful U. dianthi culture was initiated on a medium consisting of 2 g Oxoid Mycological Peptone, 2 g Difco Yeast Extract, 36 g Difco Czapek Dox Broth, and 15 g Difco Bacto Agar per litre of distilled water. The pH of the medium after autoclaving was 6.1. The aseptic urediospores were incubated in the dark at 16°C. The medium and incubation temperature were based on those of Williams et al. (1966), Bushnell (1968), and Singleton & Young (1968).

After urediospore germination, there followed a seemingly inactive period of over 8 weeks. During this time the cytoplasm in a number of germ-tubes remained visible, and some germ-tubes were seen to be slowly growing. Since the formation of an axenic culture seemed unlikely, regular observations ceased after 2 months. Twenty-three weeks after inoculation the urediospores were re-examined and found to be surrounded by a fungal colony. The colony was unlike most fungal growths, being a pale yellow, dome-shaped mycelial mass, 5 mm in diameter. The surface of the colony was irregular, with a number of comparatively large spine-like projections. At its margin branched, hyaline, septate hyphae were growing out over the agar surface (Fig. 4.1; P1, 4.1). Protruberances resembling clamp connexions were seen between some hyphal cells. These however, were

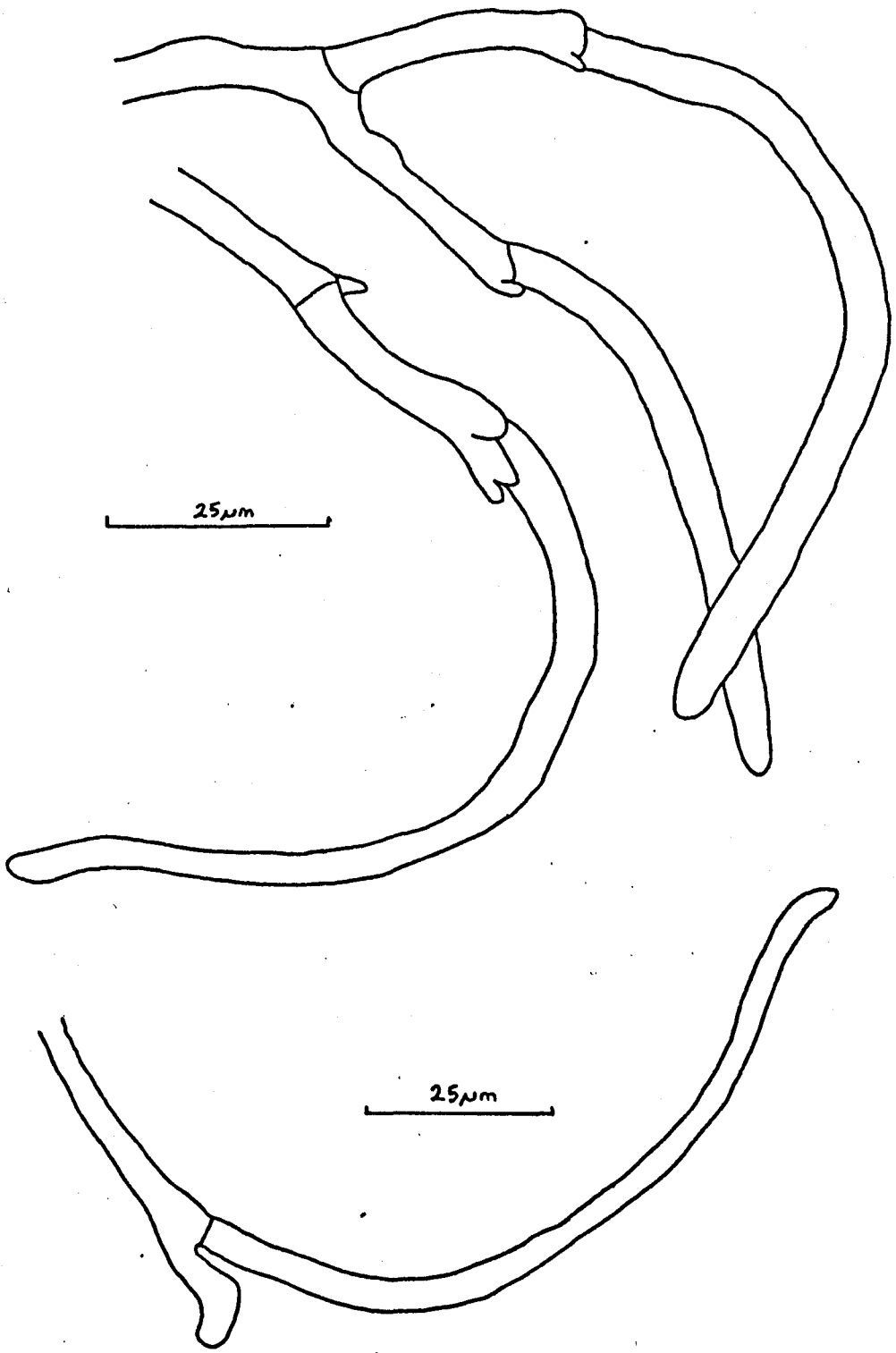


Fig. 4.1. Colonizing saprophytic hyphae found at the margin of a U. dianthi axenic culture.

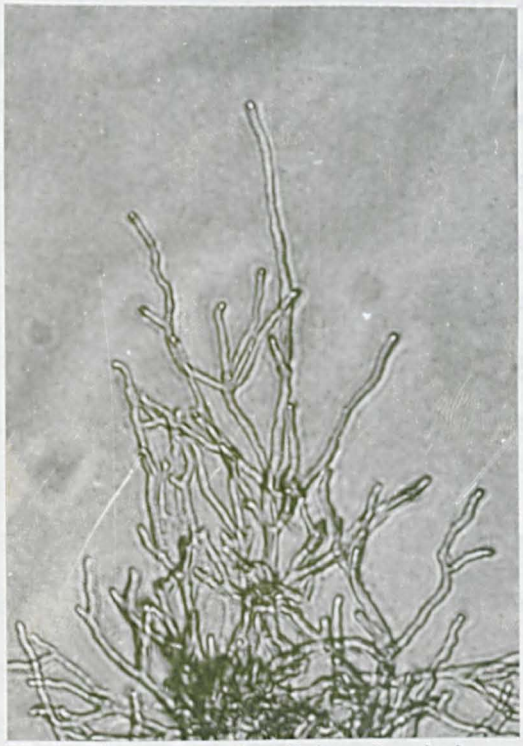


Plate 4.1. U. dianthi colonizing saprophytic hyphae growing over the surface of medium 'A' (pH 6.3) at 18°C. X 290.

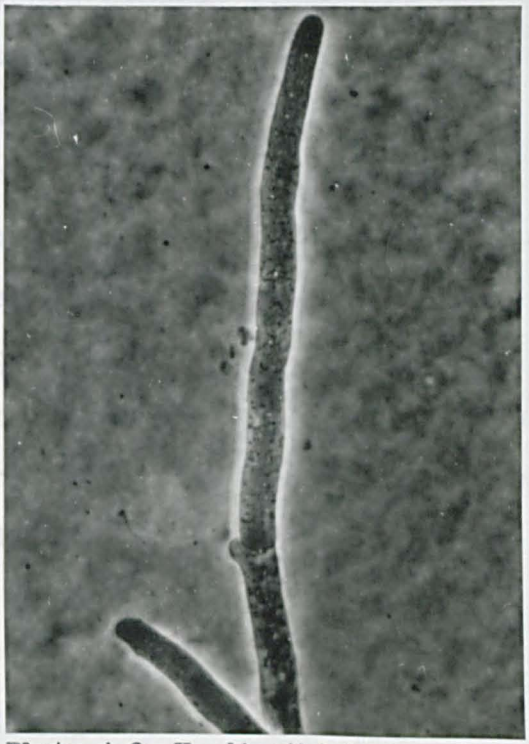


Plate 4.2. U. dianthi hypha with a lateral branch growth resembling a clamp connexion (on medium 'N', pH 6.0, 18°C). X 960.

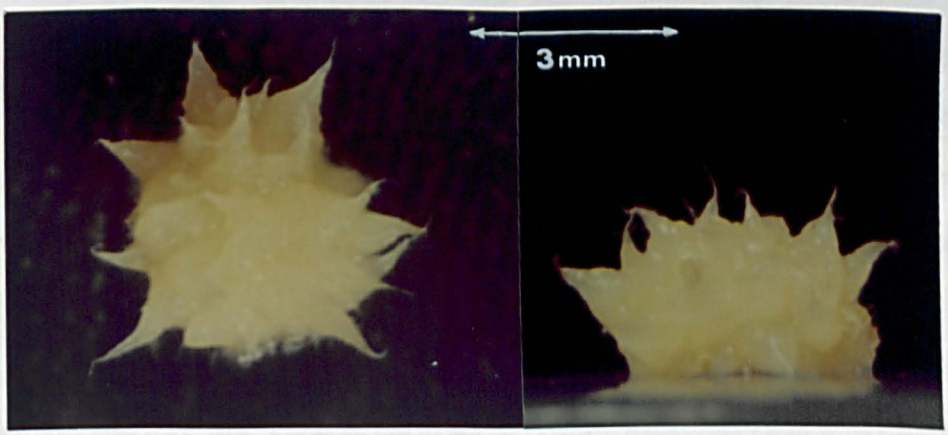


Plate 4.3. U. dianthi colony (43 days old) growing on medium 'D' at 18°C.

thought to be small developing hyphal branches. (Pl. 4.2).

The colonizing saprophytic hyphae closely resembled the intercellular parasitic hyphae found in freeze microtome sections of infected carnation leaves. The mass of the colony consisted of densely interwoven and highly branched hyphae (Fig. 4.2). Small knob-like branches which resembled haustoria were seen. Similar projections were found in some axenic cultures of P. graminis f. sp. tritici race 126 - ANZ - 6, 7, by Williams (1969). Fixing in acetic acid - ethanol and staining with Giemsa revealed that the hyphal cells possessed two nuclei (Fig. 4.3). A very large proportion of the colony grew above and on the agar surface, but some hyphae did grow into the agar. (Fig. 4.4).

4.31.2 Initial sub-culture experiment.

Fragments of the original colony 0.5 - 1.0 mm in diameter were successfully subcultured on two other peptone-yeast extract media, 'A' (pH 6.10) and 'B' (Table 4.1). These subcultures were incubated at 18°C in the dark. Usually they required 1 - 2 weeks to establish themselves and begin active growth. Subcultures from colonies growing on medium 'A' were later established on media 'C', 'D' (Pl. 4.3) and 'E' media, and appeared to grow as well as those on media 'A' and 'B'. Subcultures from colonies on medium 'E' have also been established on media 'F', 'G' and 'H' (Table 4.1; Pl. 4.4). These media contained yeast extract, peptone or casein hydrolysate as single supplements. Oxoid Czapek Dox Agar (pH 5.9 and pH 6.4) alone did not support the growth of U. dianthi.

Soon after subculturing, the young colonies appeared white in colour. As the cultures grew larger, the colour changed to yellow and then to pale orange. On medium 'A' (pH 6.1) at 18°C this process took about 5 weeks. Optimum

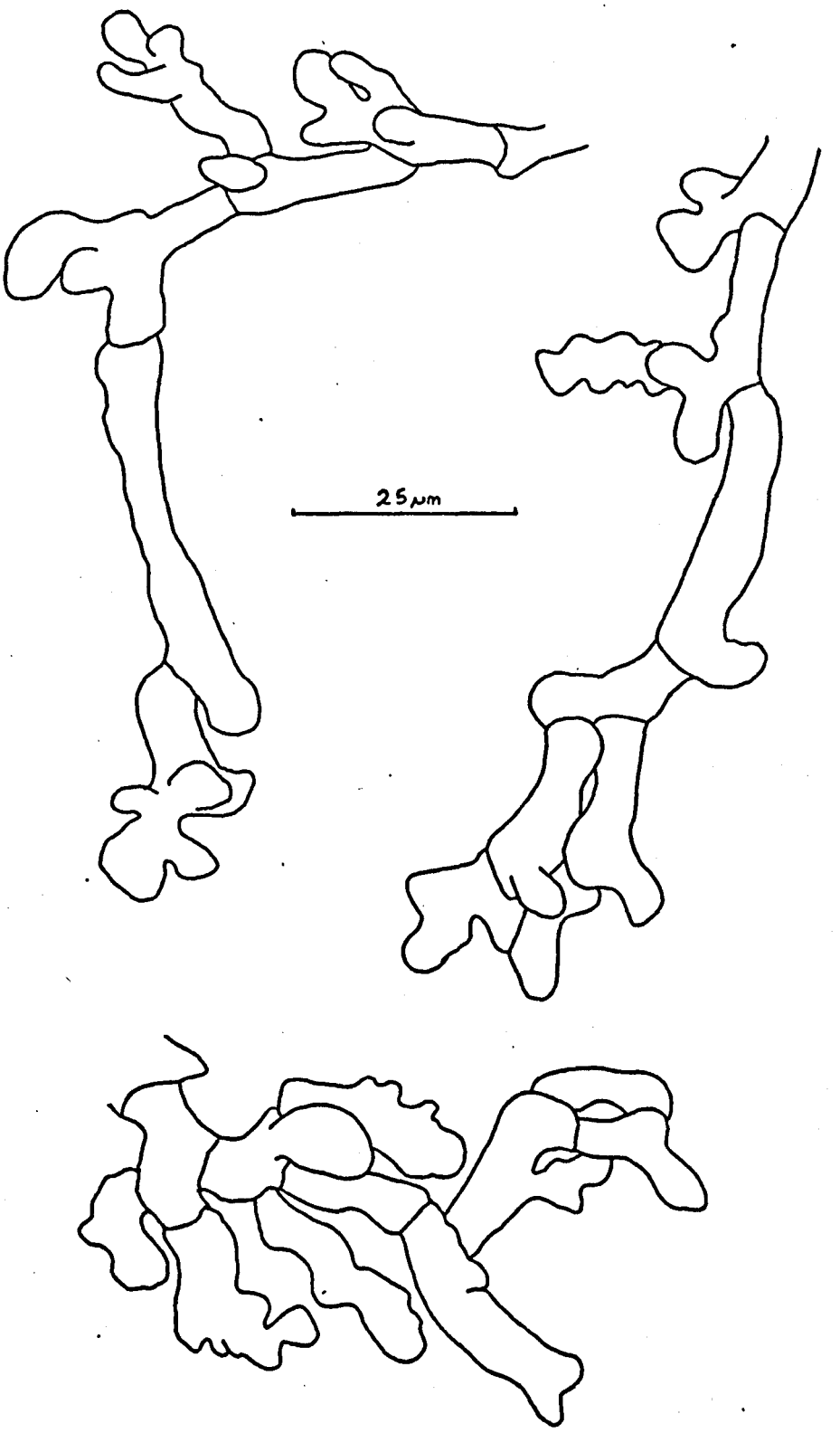


Fig. 4.2. U. dianthi saprophytic hyphae from a colony centre.

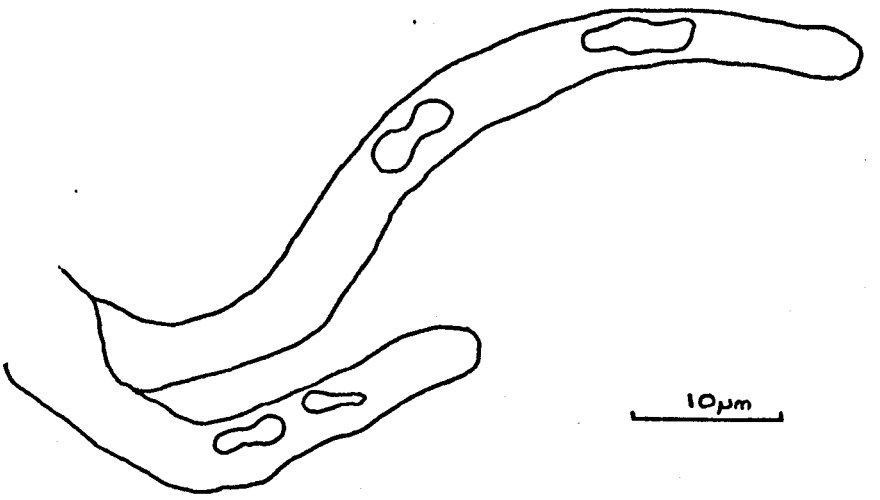


Fig. 4.3. U. dianthi saprophytic hyphal cells showing nuclei.

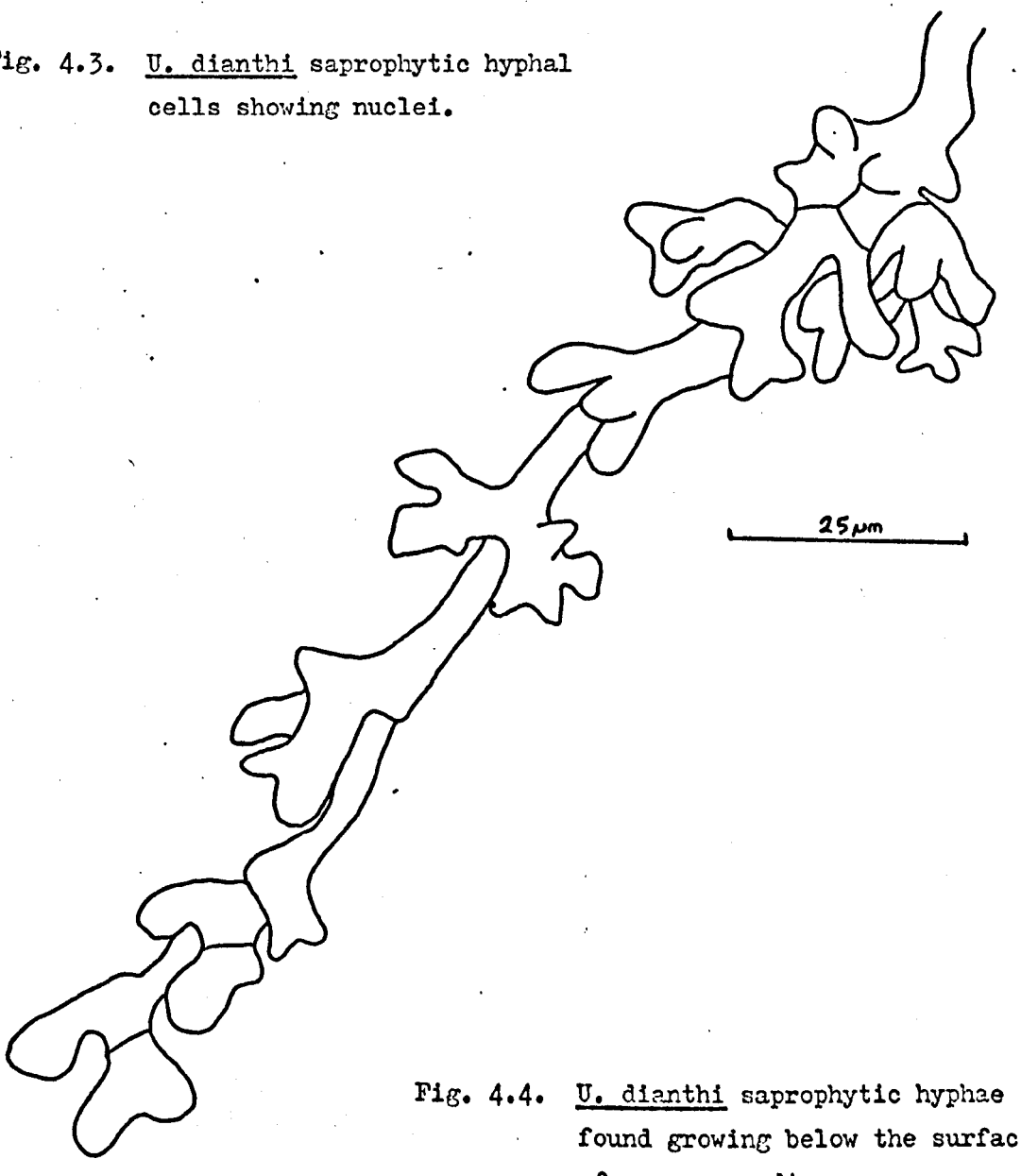


Fig. 4.4. U. dianthi saprophytic hyphae found growing below the surface of an agar medium.

TABLE 4.1 Culture media (values in g/1 of distilled water).

Medium code letter	BASAL MEDIUM			YEAST EXTRACT		PEPTONE		CASEIN HYDROLYSATE		pH
	Difco Czapek Dox Broth	Difco Bacto Agar	Oxoid Czapek Dox Agar	Difco	Oxoid	Oxoid Evans' Mycological		British Drug Houses	Oxoid	
A	36	10	-	2	-	2	-	-	-	-
B	36	10	-	1	-	1	-	-	-	6.10
C	36	10	-	2	-	2	-	2	-	6.20
D	-	-	45	-	2	-	2	-	2	6.30
E	-	-	45	-	2	-	2	-	-	6.30
F	-	-	45	-	2	-	-	-	-	6.40
G	-	-	45	-	-	-	2	-	-	6.40
H	-	-	45	-	-	-	-	-	2	6.50
I	36	10	-	2	-	-	-	2	-	6.10
J	36	10	-	-	-	2	-	2	-	6.10
K	36	10	-	2	-	-	-	-	-	6.10
L	36	10	-	-	-	2	-	-	-	6.05
M	36	10	-	-	-	-	-	2	-	6.10

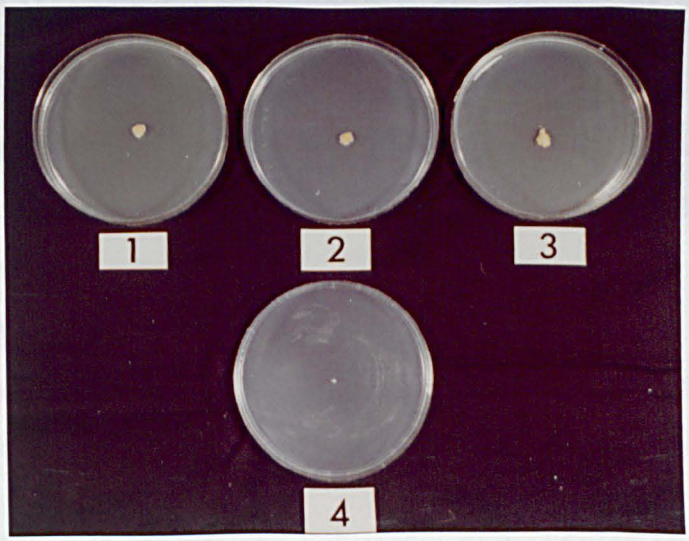
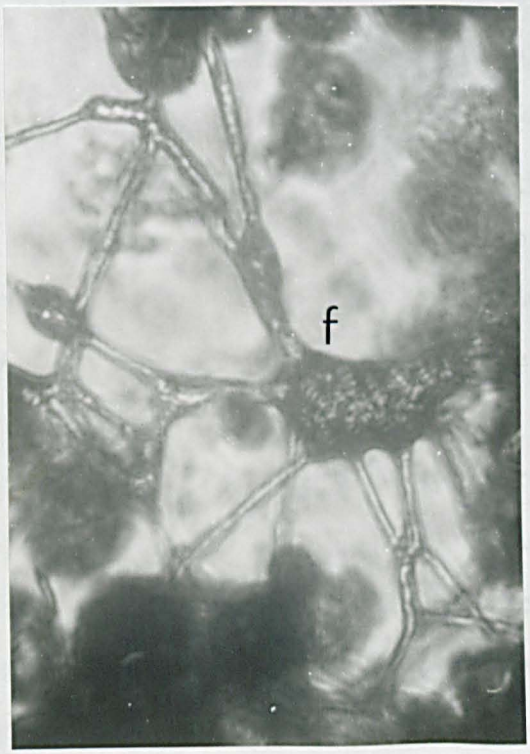
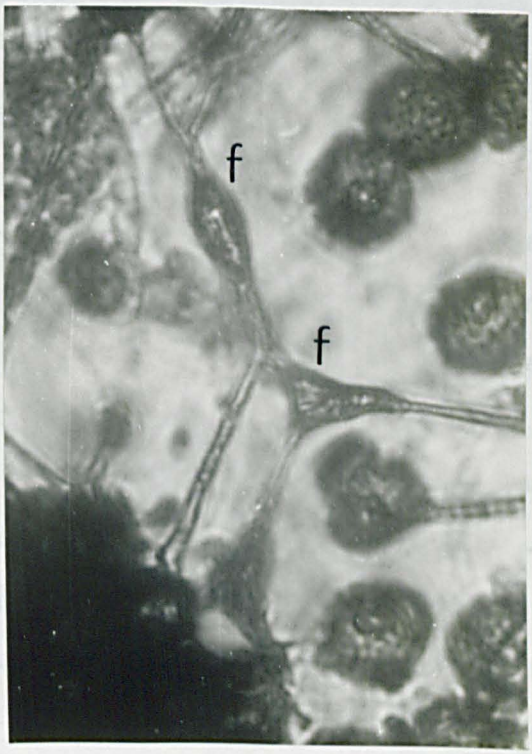


Plate 4.4. U. dianthi colonies on media 'F' (1), 'G' (2) and 'H' (3). No growth occured on Oxoid Czapek Dox Agar at pH 6.4 (4).





Plates 4.5-6. Fusion bodies (f) formed from U. dianthi urediospore germ-tubes on medium 'A' (pH 5.8). Plate incubated at 16°C for 11 days. X 520.

growth of subcultures was obtained at 18°C - 20°C on agar media of pH 5.8 - 6.2.

U.dianthi was also successfully sub-cultured in 25 ml of a liquid medium (medium C minus agar) at pH 6.1. The fungus was incubated at 18°C in 100 ml flasks plugged with cotton wool. Although submerged, the subcultures established themselves and formed spherical colonies.

4.31.3 Further initiation experiments.

In further experiments, vegetative growths of U. dianthi were initiated from spores on medium 'A' between pH 5.4 and 6.3. At 18°C development of saprophytic hyphae began 6 - 8 weeks after germination. At 16°C and 20°C this process took 7 - 13 weeks. No axenic cultures developed at 22°C.

In another series of experiments, colonies were initiated from spores at 18°C on media 'I', 'J', 'K', 'L' and 'M' (Table 4.1).

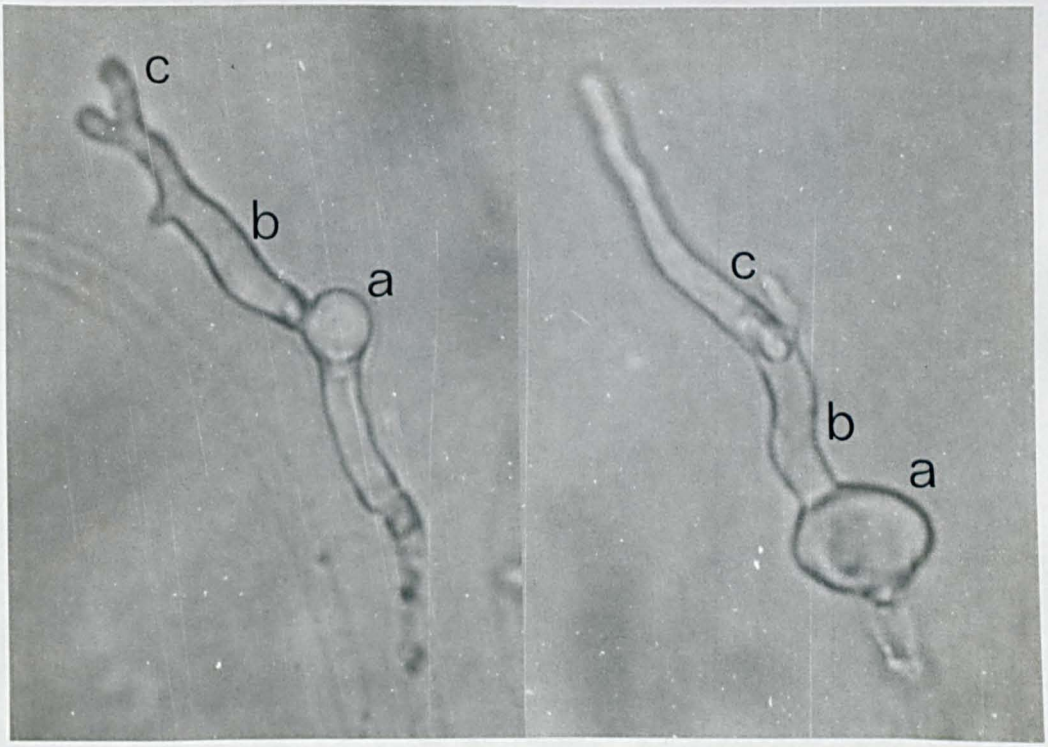
Development of saprophtic hyphae took 5 - 6 weeks except on medium 'L', where 11 - 13 weeks were needed.

4.31.4 Process of initiation of saprophytic growths.

The first urediospores germinated 1 - 2 h after inoculation on the agar medium. Germination was usually complete within 24 h, but this depended on the number of spores present as at high concentrations there was an inhibition of germination. After 48 h normal germ-tube growth has ceased. Some germ-tubes burst at their apex, discharging their contents on the agar. Fusions between germ-tubes occured in areas of high spore density. Aggregations of germ-tube protoplasm were also formed not far above the agar surface (Pl. 4.5 & 4.6). These aggregations closely resembled the urediospore germ-tube 'fusion bodies' described by Rodenhiser & Hurd-Karrer (1947). They seemed to bind crossing hyphae together, to withdraw cytoplasm

from the hyphae and pull them taut. In some instances appressoria were formed, from which structures resembling substomatal vesicles and infection hyphae developed (Pl. 4.7 & 4.8; Fig. 4.5). The cytoplasm in the majority of germ-tubes and infection hyphae became disorganised and they ceased to grow. A number of germ-tubes however, continued to grow slowly over the agar surface. After a few days, aerial hyphae usually began to develop from these tubes. These hyphae were about 3 μm in diameter and initially possessed very few septa. Urediospores seeded on medium 'L' at 18°C gave rise to unusually profuse growths of aerial hyphae, some up to 1.50 mm in length (Pl. 4.9). On one 'A' medium (pH 5.8) plate, an aerial hypha was found which terminated in a structure with thickened walls (Fig. 4.6). Aggregations of closely associated branched, anastomosing aerial hyphae developed in places. A number of aerial hyphae grew downwards to the agar surface.

After a seemingly inert period or 'lag phase' of 4 - 13 weeks (depending on temperature, medium, and pH), hyphae which colonized the agar surface and formed the mass of the colony were produced in certain areas of high spore concentrations. These colonial hyphae were 4 - 7 μm in diameter, highly branched, regularly septate and often bore knob-like projections; they resembled those found in the original colony and in subsequent subcultures. Microscopic examination during the 'lag phase' revealed hyphae of slightly differing morphology in both aerial and agar surface growth (Fig. 4.7). This suggested that the initial hyphae slowly differentiated to the colonial form hyphae through various transitionary types. Colonial hyphal cells were in fact observed developing from morphologically intermediate hyphae, which were rather more highly septate and branched than the initial hyphae. Some



Plates 4.7-8. Structures resembling appressoria (a), sub-stomatal vesicles (b) and developing infection hyphae (c) formed from U. dianthi urediospore germ-tubes after 11 days incubation at 16°C on medium 'A' (pH 5.8). X 800.

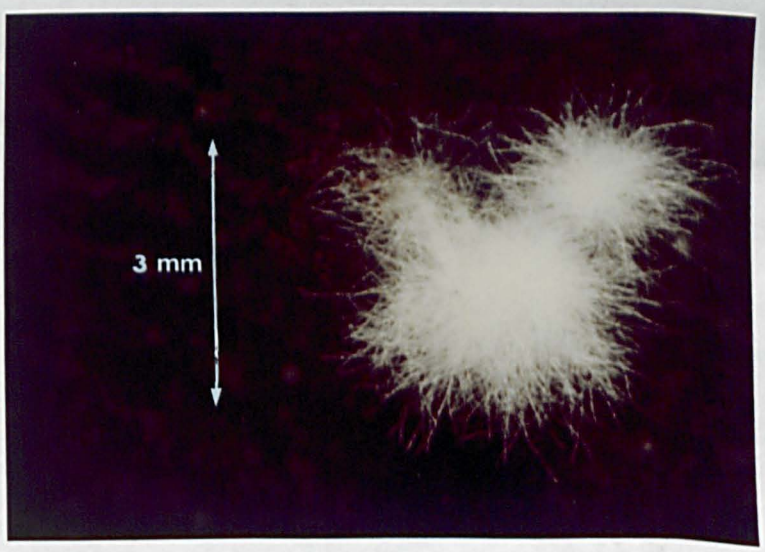


Plate 4.9. Aerial hyphae 45 days after U. dianthi urediospore inoculation on medium 'L' (plate incubated at 18°C).

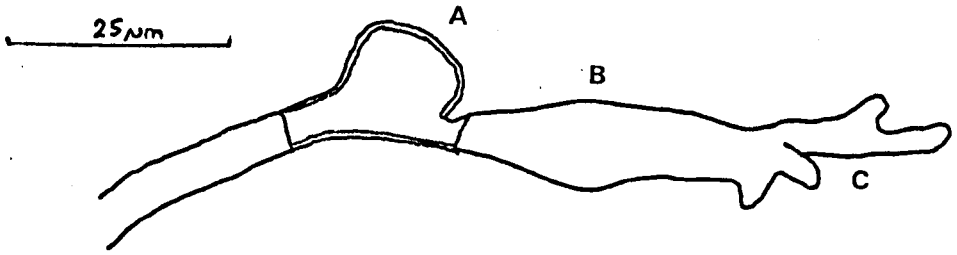


Fig. 4.5. U. dianthi saprophytic hyphal structures resembling an appressorium (A), sub-stomatal vesicle (B), and developing infection hyphae (C).

Fig. 4.6. U. dianthi saprophytic aerial hypha terminating in a structure with thickened walls.

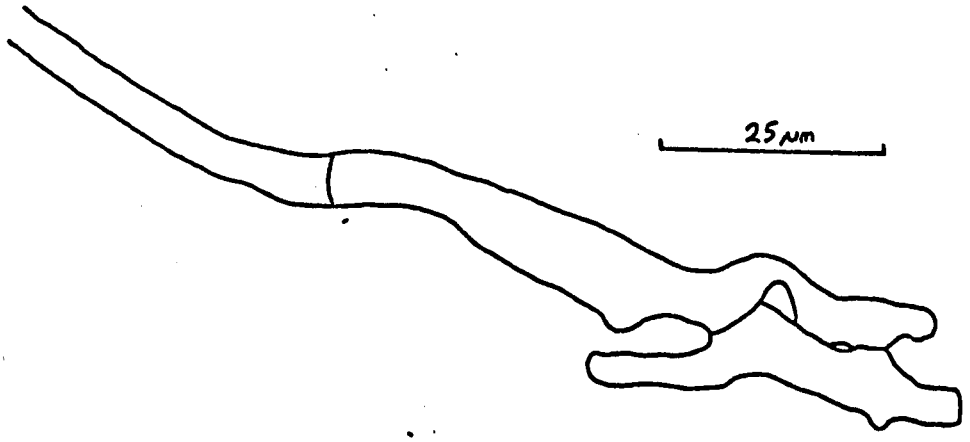


Fig. 4.7. A differentiating U. dianthi saprophytic hypha.

continuously observed differentiating hyphae failed to evolve into the successful colonizing form (Pl. 4.10), but this may have been due to the powerful light source used in the microscope killing the sensitive hyphae.

Once initiated, successful saprophytic colonies spread relatively rapidly. A subculture developing on 'A' medium (pH 6.3) at 20°C attained a diameter of 3 mm after 3½ weeks incubation. Growth of colonies usually stopped after 2 - 3 months.

4.31.5 Spore-like structures found in the mycelium.

Williams et al. (1967) reported the formation of urediospores and teliospores on P. graminis f. sp. tritici cultures growing on media containing 0.1% Evans' Peptone. U. dianthi cultures did not form urediospores or teliospores on any of media used. Angularly globose hyphal cells, usually 16 - 25 µm in diameter with colourless walls 0.5 - 2 µm thick, were however fairly common in the mycelium of older colonies (Fig. 4.8). They were also formed at the margins of colonies transferred to slightly unfavourable media or exposed to a light source. These cells resembled aeciospores. When separated from old colonies and placed on new media, they were observed to germinate and initiate new growths of colonial saprophytic hyphae (Pl. 4.11 and 4.12). Under the phase-contrast microscope most of these spore-like cells were seen to possess two nuclei. Small pieces of hyphae (minimum one cell) also seemed capable of initiating colonies (Pl. 4.13). When old colonies were dragged over agar surfaces, saprophytic growths developed where cells and the spore-like structures were detached (Pl. 4.14).

4.31.6. Carnation re-infection attempts.

In the absence of suitable spores, attempts were made to

Plate 4.10. X 240.

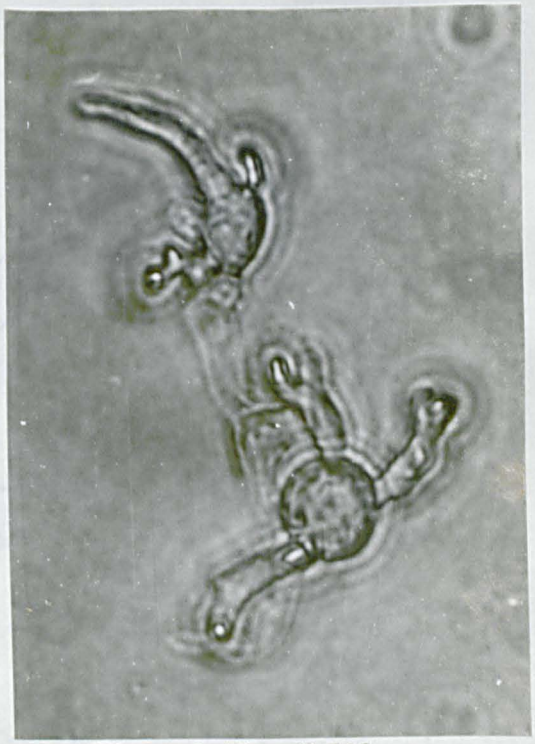


Plate 4.11. X 580.

Plate 4.10. U. dianthi filamentous saprophytic hypha differentiating to the branched colonial form after 43 days incubation at 18°C on medium 'J'.

Plates 4.11-12. U. dianthi saprophytic hyphae growing from spore-like cells after 7 days incubation at 18°C on medium 'A' (pH 6.05).

Plate 4.13. U. dianthi saprophytic hyphae developing from a small hyphal fragment after 7 days incubation at 18°C on medium 'A' (pH 6.05).

Plate 4.12. X 580.

Plate 4.13. X 580.

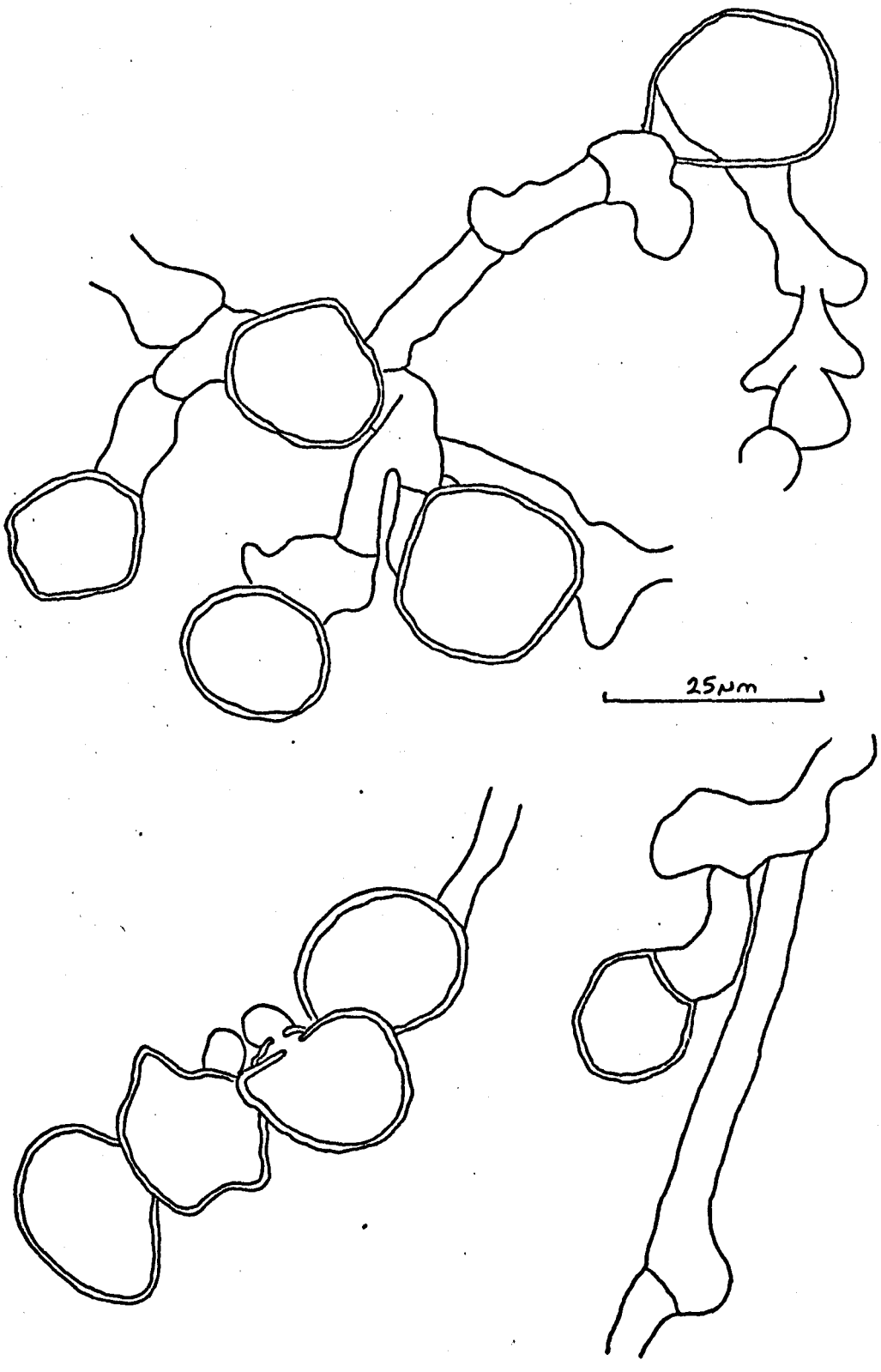


Fig. 4.8. Spore-like cells formed in older U. dianthi saprophytic colonies.

Plate 4.14. Saprophytic growths of U. dianthi initiated from spore-like cells and hyphal fragments (detached from an old colony when dragged across the surface of a 'L' medium agar) after 40 days incubation at 18°C.

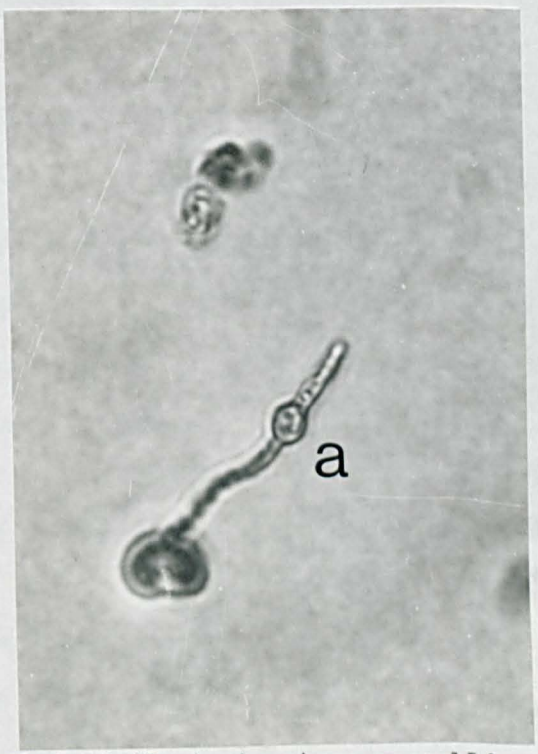


Plate 4.15. A structure resembling an appressorium (a) formed on medium 'A' (pH 6.0) 2 days after P. arenariae basidiospore inoculation and incubation at 20°C. X 560.

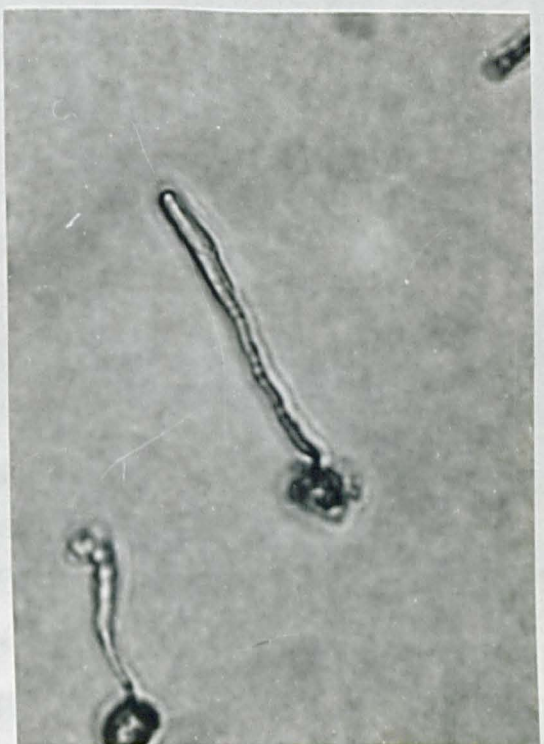


Plate 4.16. The increased diameter of a P. arenariae basidiospore germ-tube 14 days after inoculation on medium 'A' (pH 6.0) and incubation at 20°C. X 560.

infect growing and detached susceptible carnation leaves with portions of saprophytic U. dianthi colonies.

(a) Growing leaf. A number of leaves on an actively growing young D. caryophyllus var. Grenadin Scarlet plants were selected for the re-infection attempt. The upper epidermis of the leaves were partially sterilised with alcohol and then ruptured with a sterile scalpel. Pieces of the growing margins of U. dianthi colonies were then placed on the exposed mesophyll cells. 'Parafilm' membrane was used to seal the wound. The young carnation was then placed in a desiccating chamber with its roots in water. The desiccator was placed in an environmental cabinet (12 h day) at 18^{0}C (optimum for rust growth in vitro). After one week, the carnation was removed from the desiccator and placed in a glasshouse.

(b) Detached leaf. Young leaves were detached from D. caryophyllus var. Grenadin Scarlet plants and placed in a Tween 80 solution for 10 min. The leaves were then sterilized by the same procedure as before (aseptic urediospore technique). The epidermis of the leaves was ruptured in sterile water by squeezing with sterile forceps. The leaves were then placed in Petri dishes containing U. dianthi cultures (growing on 'A' media,pH 6.0) with the ruptured region lying on the growing margin of the fungal colony at 18^{o}C in the dark.

Both attempts were unsuccessful.

4.32 Results of P. arenariae initiation attempt.

P. arenariae basidiospores were discharged over various 'A' media of pH's,5.8,6.0,6.2, and 6.5. Although no axenic cultures of P. arenariae were initiated, appressoria were observed to form on the agar surface in some places (Pl. 4.15). On one plate ('A' medium, pH 6.0) it was estimated from a count of 500 germ-tube growths, that approximately 15% developed appressoria. It was noted that further development of the germ-tube stopped at or soon after appressorium formation. A few germ-tubes from the remaining 85% however slowly grew out across the agar surface.

It was noted that the diameter of the germ-tube increased (Pl. 4.16). After remaining alive for several weeks, the cytoplasm in these tubes eventually died.

4.4 DISCUSSION

The saprophytic growth of U. dianthi upon media similar to those used by other workers confirms the suitability of yeast extract, peptone, or casein hydrolysate, together with sucrose and Czapek Dox minerals, for culturing rusts. The essential nutrients or growth factors provided by the complex organic supplements must be present in yeast extract, in peptone and also in casein hydrolysate.

Bushnell (1968) working with P. graminis f. sp. tritici obtained saprophytic growth only in areas of high inoculum density (100-200 spores/mm^2). It has been suggested (Scott & Maclean, 1969) that metabolites leaking from germinating urediospores are essential for saprophytic initiation and that usually only in regions of high spore densities are they found in effective concentrations. Microscopic examination has shown that more protoplasm is present in germ-tubes closely associated with spore clusters than in germ-tubes from isolated and scattered urediospores.

The extended periods of incubation necessary for the initiation of the colonial hyphae indicate a slow metabolic adaption of the rust to its new environment. This process, as suggested by Scott & Maclean (1969) may involve a change in a fungal genome from a parasitic to a saprophytic expression. This could explain why the reinfection experiments were unsuccessful. Such a genome change could possibly result from nuclear interchanges following the fusions of germ-tubes and hyphae (cytoplasm and possibly nuclei have been observed by Little and Manners (1969) to pass between fused urediospores germ-tubes of P. striiformis) or by chromosomal exchange during dikaryotic mitosis (Hartley & Williams, 1971b).

The metabolic adaption to the artificial environment

seems to be associated with a gradual morphological change in the hyphae. This suggests a link between metabolism and morphological form; it is only when metabolising successfully (fully adapted to their environment) that the hyphae have a stable morphology. The gradual morphological change indicates the possibility of a step by step change in the rust genome.

Scott & Maclean (1969) classified the various types of saprophytic growth of P. graminis f. sp. tritici into three groups. In the first group, axenic initiation began soon after urediospore seeding, and the colony either staled or sporulated, growth ceasing after 2 - 4 weeks. The growth of U. dianthi resembles their second group, in that the rust needed extended periods of incubation before it formed colonies and that vegetative colonies would be maintained indefinitely by subculture. (U. dianthi colonies were subcultured for two years). It differs in that at no time were urediospores or teliospores produced. The spore-like cells resembling aeciospores however, were formed extensively in the mycelium of older colonies. The third type of growth was intermediate between the first two types, both in the time required for colony formation and in its morphology. From descriptions and photographs, the white fluffy colonies of P. graminis f. sp. tritici initiated by others workers in some respects resemble the initial aerial and surface filamentous hyphae (Pl. 4.9) formed from the germ-tubes of U. dianthi urediospores before the true colonzing hyphae have differentiated. Comparisons therefore may not be valid.

Williams (1971) found that dikaryotic mycelia of P. graminis f. sp. tritici only arose from germ-tubes which formed an infection structure (appressorium and substomatal vesicle). Dikoryotic mycelia were found by Bose & Shaw (1971) however

to develop from centres of germ-tube anastomosis without the formation of infection structures. Work with U. dianthi indicates that infection structures are not necessary for the formation of dikaryotic saprophytic strains.

A change in the fungal genome from a parasitic to a saprophytic form has been suggested as a possible reason why U. dianthi was unable to reinfect its host. It is possible however that U. dianthi, a slow colonizer in vitro, was excluded from the leaf by wound reactions before infection of the carnation mesophyll cells was achieved.

Attempts to grow P. arenariae in vitro, although unsuccessful, were encouraging in that the cytoplasm in some of the basidiospore germ-tubes remained alive for several weeks. It was believed that axenic cultures could have been initiated if further experimentation with the composition and pH of the medium had been possible.

5.1 INTRODUCTION

Once saprophytic hyphae were initiated, it was thought desirable to investigate the conditions necessary for the optimum growth of U. dianthi in axenic culture. This involved varying the pH of the medium, the media constituents, the concentrations of media constituents, and the temperature of incubation. Work was also undertaken on the effect of autoclaving the media.

Initially, experiments were carried out with U. dianthi colonies growing directly on the agar surface. This method enabled the diameters of colonies to be accurately measured through the base of Petri dishes. Accurate weighings of the colony growths however could not be achieved, as although the colonies usually lifted off the agar cleanly and neatly (i.e. colonies in a state of active growth), there was the possibility of agar particles clinging to the mycelium. Also some growth into the agar did occur, and this could not be separated and weighed. This was thought to amount to between 1 and 5% of the total colony weight. The results from this weighing method, though not accurate, were thought to be comparable.

Most experiments were repeated after the initial agar surface growth attempts using the Millipore filter disc method (see 5.22.2). Here the colony grew on a filter disc resting on the agar surface. The filter permitted nutrients to move through from the agar, but prevented the colony hyphae from growing into the agar. The Millipore method enabled the weights of the colonies to be accurately determined as :- 1) All the rust colony was above the agar surface and no unweighable mycelium was present in the agar. 2) There was no possibility of agar clinging to the mycelium and being included in the dry weight.

5.2 MATERIALS AND METHODS.

5.21 Standard experimental procedure.

5.21.1 Agar media and pH.

Media were prepared and pH values recorded as previously described (see Chapter 4.23). All amino acids and chemicals used were of the highest purity commercially obtainable.

5.21.2 Stock cultures.

Axenic culture stocks of *U. dianthi* were originally maintained at 18°C and 20°C on a medium consisting of :-

2 g Evans' Peptone,
2 g Difco Yeast Extract,
36 g Difco Czapek Dox Broth,
10 g Difco Bacto Agar
per litre of distilled water (pH 6.0).
(Medium code 'A6')

However, later stocks were maintained at 18°C on a medium that encouraged faster mycelial growth. This consisted of :-

5 g Evans' Peptone,
36 g Difco Czapek Dox Broth,
10 g Difco Bacto Agar
per litre of distilled water (pH 6.0).
(Medium code 'N')

5.21.3 Subculturing techniques.

In all experiments, subcultures were transferred from stock cultures growing on either 'A6' or 'N' media. In each experiment subcultures were taken from *U. dianthi* colonies of the same age and approximately the same size. The subculture pieces were also of approximately the same size, varying between 1-3 mm^2, depending on the age and condition of the colonies chosen. Usually between 15 and 25 subcultures were obtained from each colony and they were either placed on the agar surface

in the centre of the Petri dish, or on the centre of a Millipore filter. If a Millipore filter was used, any agar adhering to the subculture was removed. Approximately the same amount of agar (40 ml) was poured into each Petri dish in each experiment. Deep plates were poured to slow the dehydration of the agar medium. After the subculturing procedure (see Chapter 4.21.2), all Petri dishes were sealed with adhesive tape before being taken out of the sterile inoculating room. If a change from the stock culture temperatures of 18°C and 20°C was necessary in an experiment, the subcultures were placed initially for 48 h in incubators of their stock culture temperatures in order that they might establish themselves. After this time, the plates were moved to the correct experimental temperature. To reduce the risk of contamination, the plates were always stored in incubators in polythene bags sealed with rubber bands.

5.22 Measurements of U. dianthi colony dry weights.

5.22.1 Agar surface colonies.

The weight of colonies growing directly on agar was determined by lifting them cleanly off the surface with a large scalpel blade, and placing them directly into porcelain crucibles of known weight. After being dried overnight at 60°C, the crucibles were cooled to room temperature over calcium chloride in a desiccator, and weighed.

5.22.2 Millipore filter disc technique.

Quick & Cross (1971) found that axenic cultures of flax rust (M. lini) could be grown on Millipore filter discs resting on the surface of an agar medium. The filter discs permitted nutrients to diffuse through, but prevented the hyphae growing into the agar. U. dianthi was successfully grown on 25 mm diameter Millipore filter discs with pore diameters of 0.45 µm (Code HAWP 02500).

The Millipore filters were sterilised by autoclaving at 120°C for 15 min. Usually twenty-five filters, with paper discs separating them, were wrapped together in a little tin foil and placed in a covered glass Petri dish, which was autoclaved. Flamed forceps were used to transfer the sterile filters to the agar surface, usually in the centre of the plate. The forceps were flamed after each transfer.

After the experiments the colonies on the filters were transferred by means of a scalpel blade to sintered glass filter crucibles (A.G. Gallenkamp Ltd.) of known weight. Distilled water washings from the upper surface of the filter discs were added, and the mycelial pads washed under suction filtration with distilled water. The filter crucibles were dried overnight at 60°C, cooled to room temperature over calcium chloride in a desiccator, and weighed.

The colonies were always actively growing when the experiments were terminated.

5.3 AXENIC GROWTH EXPERIMENTS.

5.31 The influence of pH on growth.

5.31.1 pH 5.3-7.0.

Experimental procedure. Agar media were prepared with various pH values ranging from pH 5.3 to pH 7.0. The agar was basically an 'A' medium, (see Table 4.1) but contained 15 g Difco Bacto Agar per litre of distilled water (as at low pH values, the medium failed to solidify using 10 g agar per litre). For every pH value, six plates were poured. After subculturing from 'A6' medium stocks (at 18°C) directly on the agar surface, the cultures were incubated at 18°C for 15 weeks. The diameters of the colonies were then calculated (see Chapter 4.21.3) and their dry weights found (see 5.22.1).

Results. A number of subcultures did not establish themselves on the media, especially those of the extreme pH values. Averages were calculated from the plates containing colonies. No colonies established themselves at pH 5.3 and pH 7.0, indicating that the pH limits for the axenic growth of U. dianthi were between pH 5.3-5.5 and pH 6.8-7.0. Initial observations indicated that optimum growth occurred on the medium of pH 6.0 (P1. 5.1). This was confirmed from the average diameter and weight values (Table 5.1, 5.2, Fig. 5.1).

5.31.2 pH 5.8-6.2.

Experimental procedure. In the previous experiment, the weights of the colonies, though comparable, were not accurate. This experiment was carried out to find the optimum pH growth value in the pH range pH 5.8-6.2. 'N' agar media (see 5.21.2) were prepared with pH values 5.8, 5.9, 6.0, 6.1 and 6.2. For every pH value, ten plates werepoured. Subcultures from 'N' medium stocks were placed on Millipore filter discs and incubated at 18°C for 50 days. After this time, the accurate

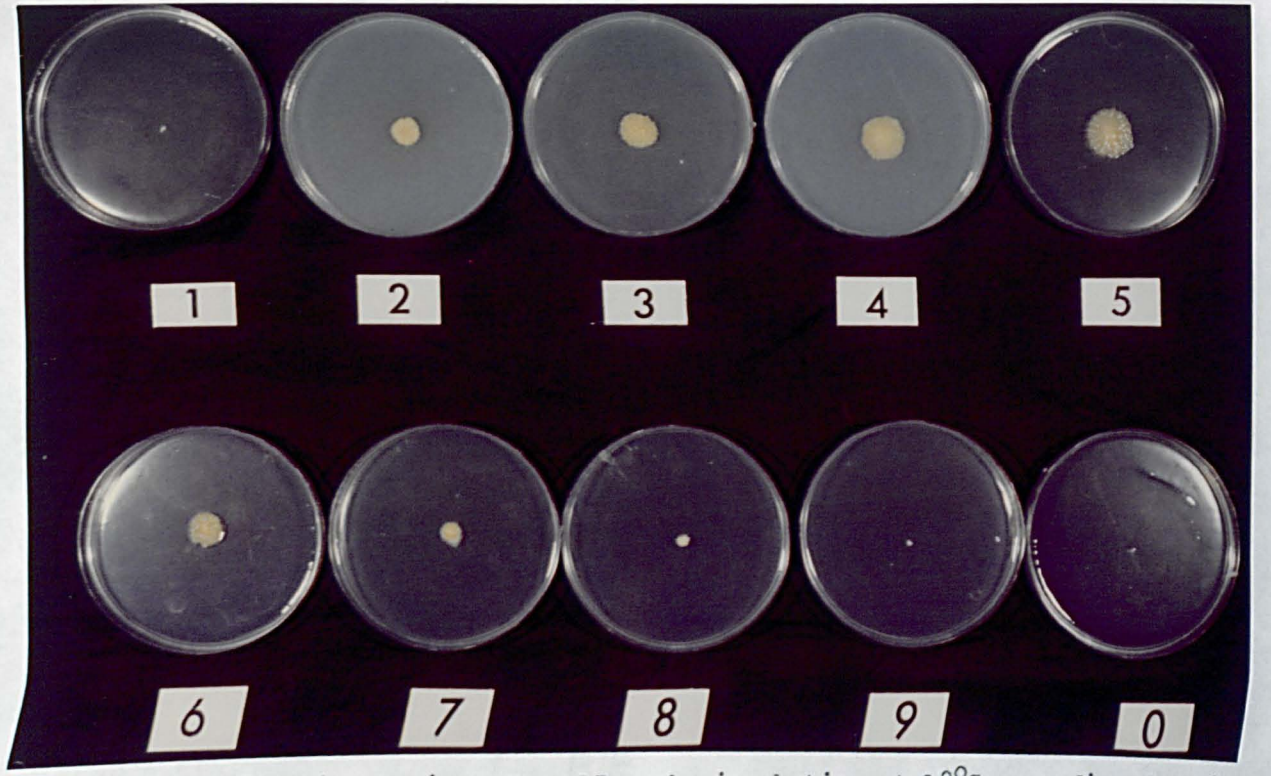


Plate 5.1. U. dianthi colonies after 15 weeks incubation at 18°C on media of various pH values.

Key. 1, pH 5.3; 2, pH 5.5; 3, pH 5.7; 4, pH 5.9; 5, pH 6.0
6, pH 6.2; 7, pH 6.4; 8, pH 6.6; 9, pH 6.8; 0, pH 7.0.

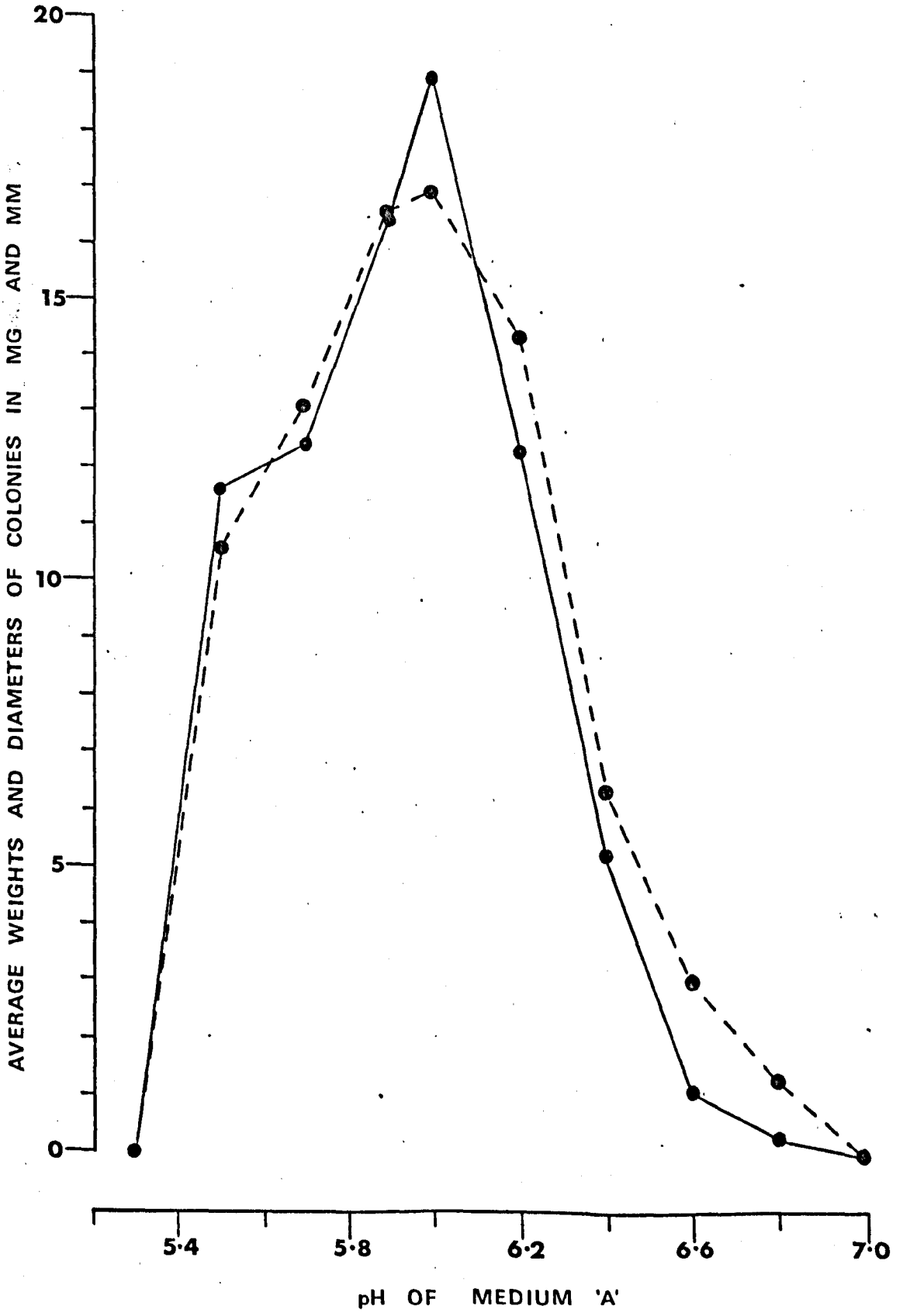
TABLE 5.1 The influence of pH (5.5-6.8) on U. Dianthi colony diameters (15 weeks incubation at 18°C).

pH of Agar Medium	Colony Diameters in mm						Averages in mm
5.5	12.5,	9.0,	10.0,	11.0,	-	-	10.6
5.7	14.0,	13.5,	14.0,	11.5,	11.5,	14.0.	13.1
5.9	16.0,	16.0,	19.0,	16.0,	16.0,	16.0,	16.5
6.0	15.0,	21.0,	15.0,	16.5,	17.0,	-	16.9
6.2	13.0,	13.0,	15.0,	16.5,	13.0,	15.0,	14.3
6.4	6.0,	4.5,	7.0,	7.5,	-	-	6.3
6.6	2.0,	3.0,	2.5,	4.5,	-	-	3.0
6.8	1.5,	2.0,	2.0,	-	-	-	1.3

TABLE 5.2 The influence of pH (5.5-6.8) on U. dianthi colony weights (15 weeks incubation at 18°C)

pH of Agar Medium	Colony weights in mg						Averages in mg
5.5	13.9,	9.7,	10.0,	12.1,	-	-	11.6
5.7	13.2,	13.1,	13.5,	11.3,	9.0',	14.2,	12.4
5.9	16.0,	15.4,	17.1,	15.9,	16.8,	17.1,	16.4
6.0	17.0,	22.5,	16.3,	18.5,	20.2,	-	18.9
6.2	11.7,	10.8,	13.7,	11.9,	15.1,	10.4,	12.3
6.4	5.8,	3.1,	5.7,	6.0,	-	-	5.2
6.6	0.2,	1.1,	0.3,	2.6,	-	-	1.1
6.8	0.2,	0.4,	0.2,	-	-	-	0.3

Fig. 5.1. The influence of medium pH on U. dianthi colony weights (●——●) and diameters (●- - -●) after 15 weeks incubation at 18°C.



dry weights of the colonies were determined (see 5.22.2). Statistical comparisons were made between the colony weights on the media of the various pH values using Student's 't' - test.

Results. The colony weights on pH 5.9, pH 6.0, pH 6.1, and pH 6.2 media were statistically different from one another at the 5% level of significance (exception, weights on pH 5.8 and pH 5.9 media). The optimum pH value for growth was on the pH 6.00 medium (Table 5.3, Fig. 5.2).

5.32 The influence of temperature on growth.

Experimental procedure. An 'A' medium (pH 6.0) was prepared and seventy-two plates poured. All subcultures were transferred from 'A6' medium stock cultures (at 20°C) to Millipore filter discs on the agar surfaces. Incubators were adjusted to nine different temperatures ranging from 4°C to 30°C, and eight plates placed in each. After 10 weeks, the weight of the colonies were determined (see 5.22.2). The differences in the weight of colonies at the various temperatures were compared statistically using Student's 't'-test.

Results. All weights at the various temperatures were calculated to be different at the 1% level of significance. The optimum temperature for the axenic growth of U. dianthi was indicated to be at 18°C (Table 5.4, Fig. 5.3).

5.33 Nitrogen and growth.

5.33.1 Peptone and yeast extract concentrations and growth.

Experimental procedure. Media were prepared with ranges of concentration of Evans' Peptone and Difco Yeast Extract. The basis of these media was 36 g Difco Czapek Dox Broth and 10 g Difco Bacto Agar per litre of distilled water. The concentrations of peptone and yeast extract were 0.1 g, 0.5 g, 1.0 g, 5.0 g, 10.0 g, 15.0 g and 20.0 g per litre of medium.

TABLE 5.3. The influence of pH (5.8-6.2) on U. dianthi colony weight (50 days incubation at 18°C).

	pH of the Agar Medium				
	5.8	5.9	6.0	6.1	6.2
Colony Weights in mg	19.5	20.1	30.9	23.9	20.3
	25.6	16.8	23.8	22.4	19.0
	18.5	22.5	23.5	20.4	20.3
	17.1	19.6	19.9	21.5	19.0
	21.5	17.3	22.4	20.4	19.3
	17.8	25.1	25.7	17.5	16.7
	21.2	23.0	26.0	21.2	16.8
	20.3	19.6	21.8	23.0	15.6
	15.2	25.4	24.2	22.2	21.8
	21.7	20.5	25.8	24.9	22.6
Averages	19.8	21.0	24.4	21.7	19.1

TABLE 5.4 The influence of temperature on U. dianthi colony weight (10 weeks incubation on 'A' medium, pH 6.0).

°C	Colony weights in mg	Averages
4	1.6, 2.5, 2.3, 1.9, 1.8, 2.1, 1.3, 1.7,	1.9
10	5.4, 6.6, 6.4, 4.1, 4.4, 4.1, 5.7, 5.2,	5.2
16	7.8, 7.6, 8.0, 7.7, 6.5, 7.0, 5.2, 5.7,	6.9
18	9.4, 9.0, 9.2, 8.5, 8.4, 9.4, 9.9, 11.0,	9.4
20	8.1, 7.7, 8.3, 7.5, 7.0, 8.9, 7.9, 7.9,	7.9
22	7.0, 6.3, 5.8, 6.3, 5.8, 5.8, 6.7, 7.8,	6.4
24	5.3, 6.8, 4.9, 4.0, 4.3, 4.4, 4.9, 4.3,	4.9
28	2.1, 2.5, 1.6, 2.8, 3.7, 3.9, 4.1, 2.8,	3.0
30	0.4, 0.9, 0.4, 0.5, 0.4, 0.3, 0.3, 0.4,	0.5

Fig. 5.2. The influence of medium pH (5.8-6.2) on U. dianthi colony weights.

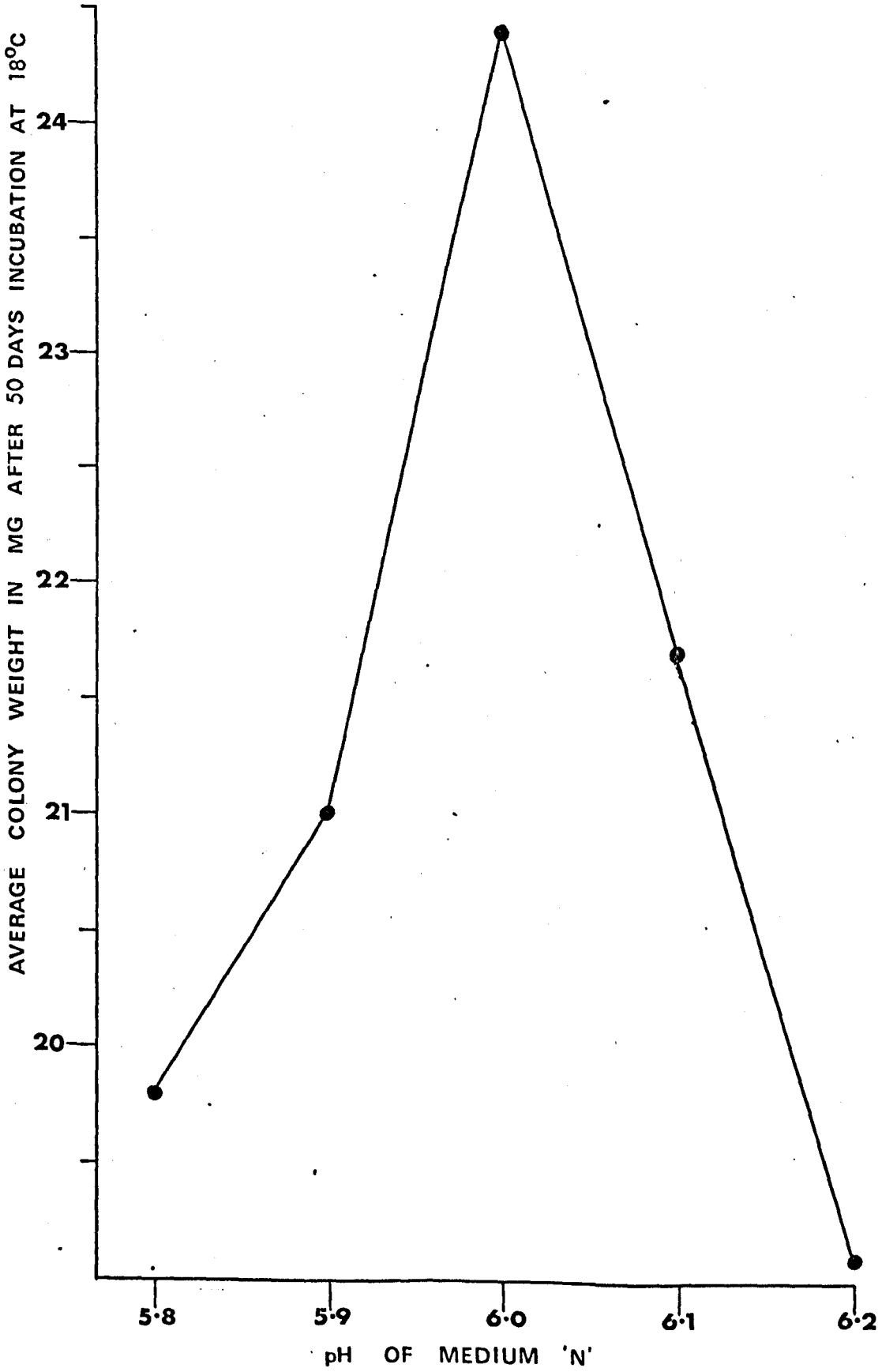
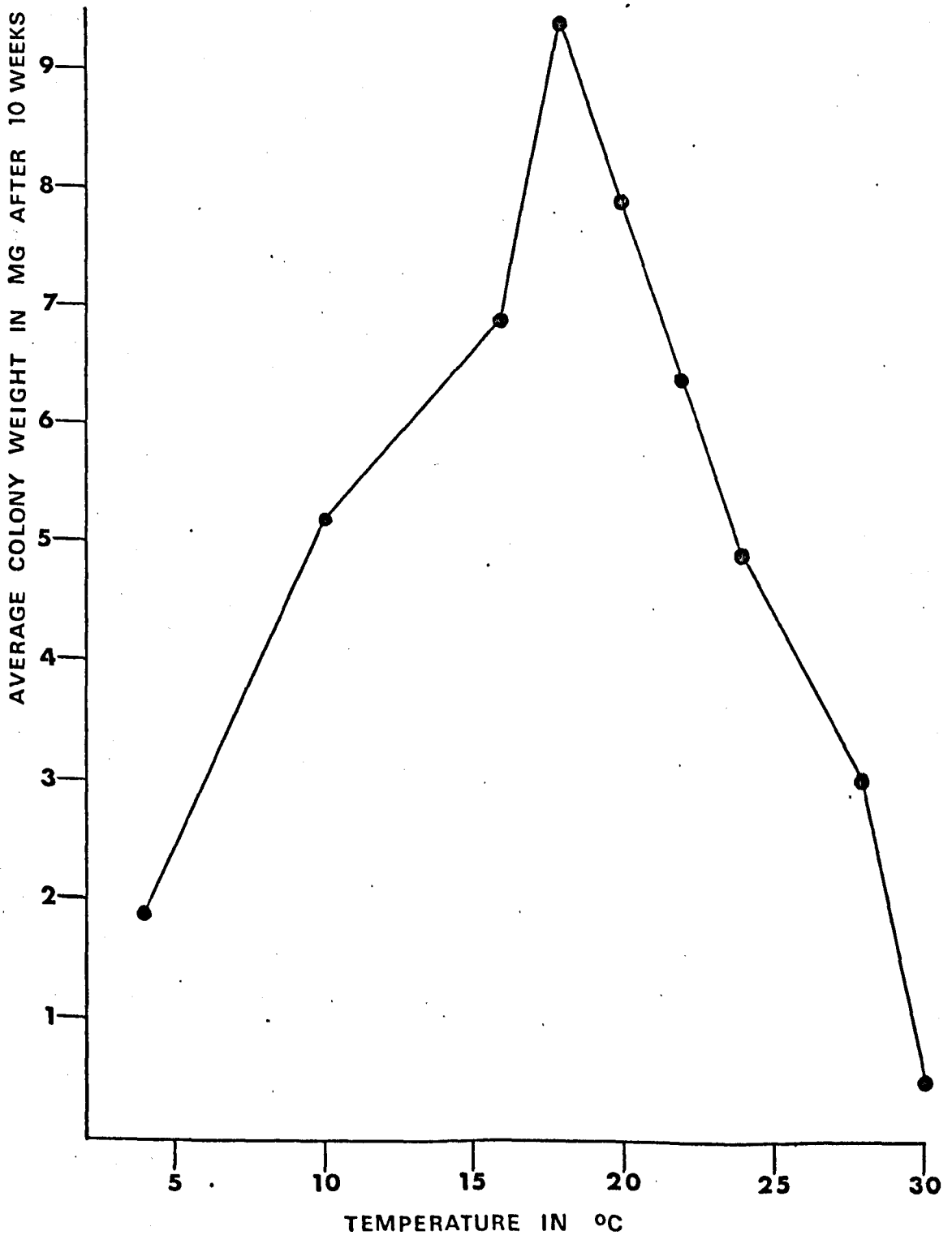


Fig. 5.3. The influence of temperature on the growth of U. dianthi colonies on 'A' medium.



Controls containing no nitrogen source were also prepared. All media were at pH 6.0 after sterilisation by autoclaving, and six plates were poured for each concentration. Fragments from 'A6' medium stocks (at 18°C) were subcultured on to Millipore filter discs. The plates were incubated for 10 weeks at 20°C, and then the colony weights determined (see 5.22.2). These were compared statistically using Student's 't'-test.

Results. The weighings indicated that the optimum growth occurred on the medium containing 10 g of nitrogen source per litre (Table 5.5, 5.6, Figs. 5.4, 5.5.). No subcultures grew on the control plates or the media containing 0.1 g and 20.0 g of nitrogen source per litre. The colony weights on the media containing different concentrations were found to be different at the 5% level of significance. Direct comparisons between the effects on growth of the peptone and yeast extract concentrations cannot be made as the two experiments were performed at different times (see Chapter 6.38).

5.33.2 A comparison of the effects of various sources or nitrogen on growth.

Experimental procedure. Media were prepared using 5 g of various organic nitrogen containing nutrients, 35 g Difco Czapek Dox Broth, and 10 g Difco Bacto Agar per litre of distilled water. The sources of nitrogen used were :-

1) Oxoid Yeast Extract
2) Difco Yeast Extract
3) Oxoid Peptone
4) Difco Bacto Peptone
5) B.D.H. (British Drug Houses) Peptone
6) Evans' Peptone
7) Oxoid Casein Hydrolysate

TABLE 5.5 Weight of U. dianthi colonies grown on media containing different concentrations of Evans' Peptone (10 weeks incubation at 20°C).

	Concentrations of Peptone per litre							
	0 g	0.1 g	0.5 g	1.0 g	5.0 g	10 g	15 g	20 g
Colony	-	-	1.7	9.2	18.6	31.4	35.1	-
Weights	-	-	1.7	7.9	25.0	31.1	26.9	-
in	-	-	1.3	7.2	22.8	29.5	29.4	-
mg	-	-	1.4	7.4	26.4	35.8	24.5	-
	-	-	1.6	8.0	27.0	27.0	27.1	-
	-	-	1.8	7.2	30.6	35.8	25.0	-
Averages	-	-	1.6	7.8	25.1	31.6	28.0	-

TABLE 5.6 Weight of U. dianthi colonies grown on media containing different concentrations of Difco Yeast Extract (10 weeks incubation at 20°C).

	Concentrations of Yeast Extract per litre							
	0 g	0.1 g	0.5 g	1.0 g	5.0 g	10 g	15 g	20 g
Colony	-	-	0.5	1.6	5.7	8.9	3.0	-
Weights	-	-	0.6	2.0	6.3	7.6	2.8	-
in	-	-	0.4	2.0	5.7	7.0	4.9	-
mg	-	-	0.4	1.9	6.4	6.8	4.3	-
	-	-	0.6	1.6	6.0	6.4	4.5	-
	-	-	0.5	1.7	6.6	7.5	2.6	-
Averages	-	-	0.5	1.8	6.1	7.4	3.7	-

Fig. 5.4. The effect of Evans' Peptone concentration on the growth of U. dianthi colonies. Weights determined after 10 weeks incubation at 20°C.

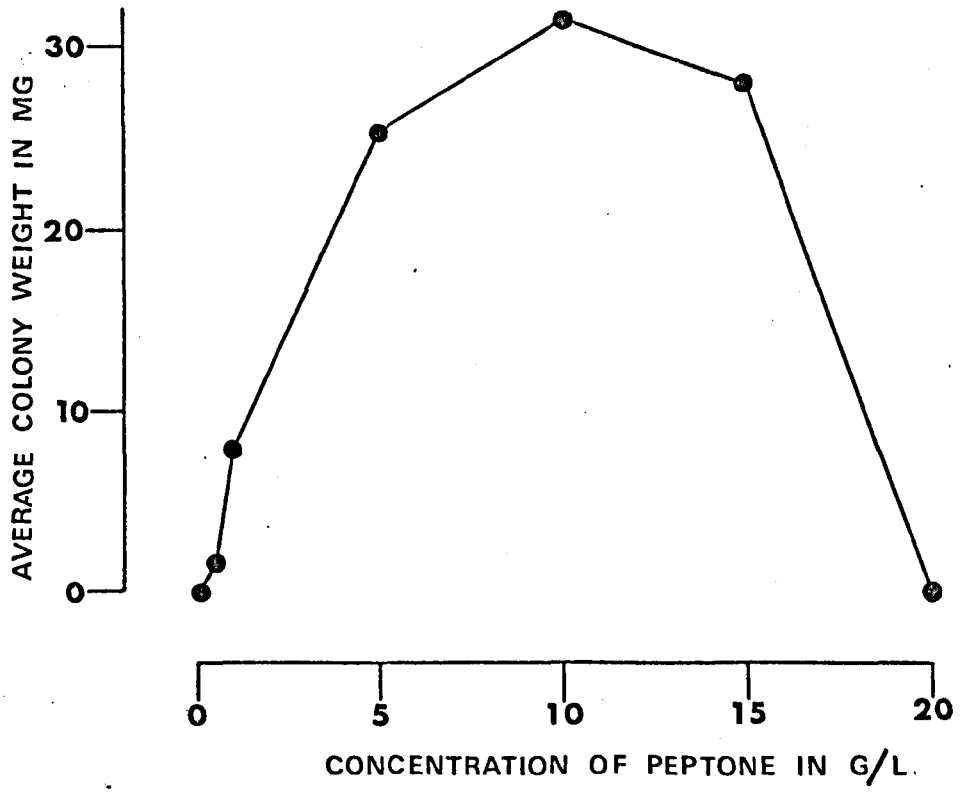
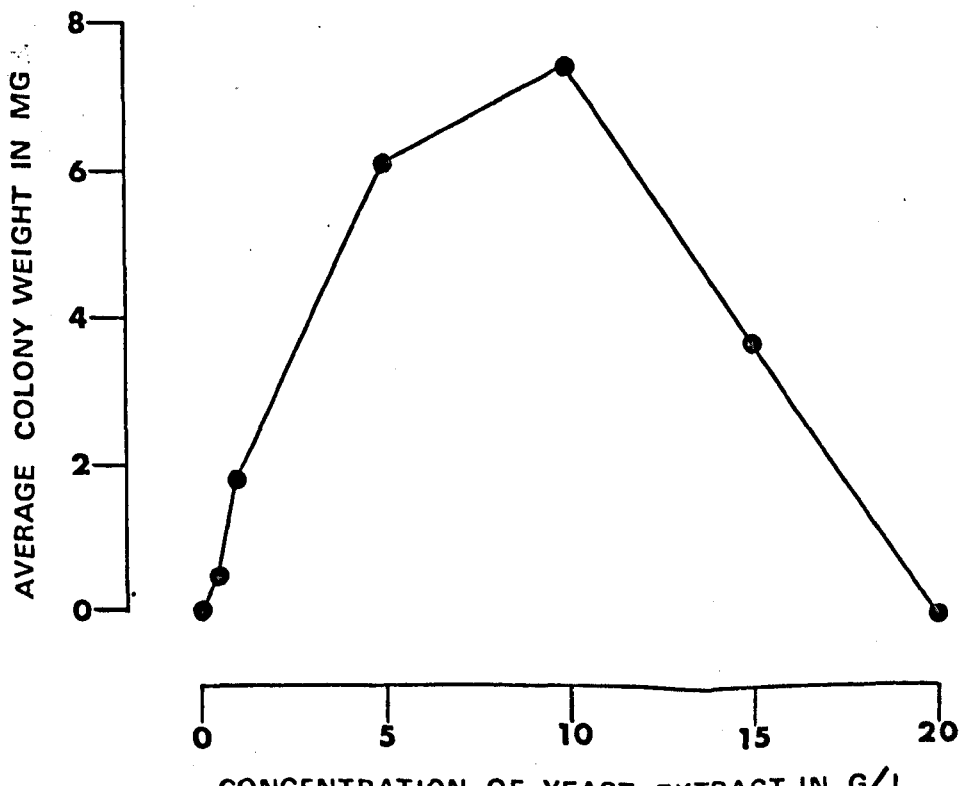


Fig. 5.5. The effect of Difco Yeast Extract concentration on the growth of U. dianthi colonies. Weights determined after 10 weeks incubation at 20°C.



8) B.D.H. Casein Hydrolysate

9) B.D.H. Casein

After autoclaving, ten plates were poured per medium. All media were found to have pH 6.0. Pieces of U. dianthi colonies growing as stock cultures on an 'A6' medium (at 18°C) were subcultured to Millipore filter discs on the plates. The subcultures were incubated for 10 weeks at 20°C and then the weight of colonies determined (see 5.22.2).

Results. Some subcultures failed to establish themselves on some of the plates. Averages were calculated from the established colonies. Evans' Peptone appeared to encourage the growth of U. dianthi more than any other nitrogen source. Difco Yeast Extract was the second best growth promoting nutrient. Casein hydrolysates were the nutrients least capable of supporting growth. No colonies were established on the medium containing B.D.H. Casein (Table 5.7, Fig. 5.6, Pls. 5.2-9).

5.33.3 The effect of autoclaving a nitrogen source (Evans' Peptone) on growth.

Experimental procedure. To find the effect of autoclaving the nitrogen source on the growth of axenic cultures of U. dianthi, two solutions of 'N' medium agar (see 5.21.2) of pH 6.0 were prepared. These were autoclaved for 5 and 20 min at 120°C. A concentrated solution of Evans' Peptone was also prepared (2.5 g /100 ml of distilled water) and its pH adjusted to 6.0. This was filtered under vacuum through a Millipore filter disc (Code VCWP 04700, 0.1 μm diameter pore) in a Millipore Sterifil filtration unit, and a sterile solution obtained. This was then added to an autoclaved solution containing 18 g Difco Czapek Dox Broth and 5 g Difco Bacto Agar per 500 ml of distilled water (pH 6.0). The resulting 'N'

TABLE 5.7. The weight of U. dianthi colonies grown on agar media containing various sources of nitrogen (10 weeks incubation at 20°C).

Nitrogen Source	Colony Weights in mg	Averages
Oxoid Yeast Extract	6.2, 6.2, 4.0, 4.5, 5.9, 6.5, 7.3, - -	5.8
Difco Yeast Extract	8.7, 7.7, 7.7, 8.2, 7.5, 8.2, - - -	8.0
Oxoid Peptone	7.6, 5.1, 5.5, 4.2, 6.5, - - - -	5.8
Difco Bacto Peptone	3.3, 2.6, 0.8, 1.3, 1.7, 3.4, - - -	2.2
B.D.H. Peptone	4.1, 3.6, 7.3, 4.2, 3.2, 7.1, - - -	4.9
Evans' Peptone	11.5, 8.7, 12.4, 8.7, 9.3, 17.2, 13.8, 21.0, 22.9	13.9
Oxoid Casein Hydrolysate	1.4, 0.8, 2.6, 1.3, 0.7, 3.2, 1.7, 2.1, 3.4, -	1.9
B.D.H. Casein Hydrolysate	0.2, 0.1, 0.1, 0.2, 0.2, 0.1, 0.2, 0.1, -	0.1
B.D.H. Casein	- - - - - - - - - -	-

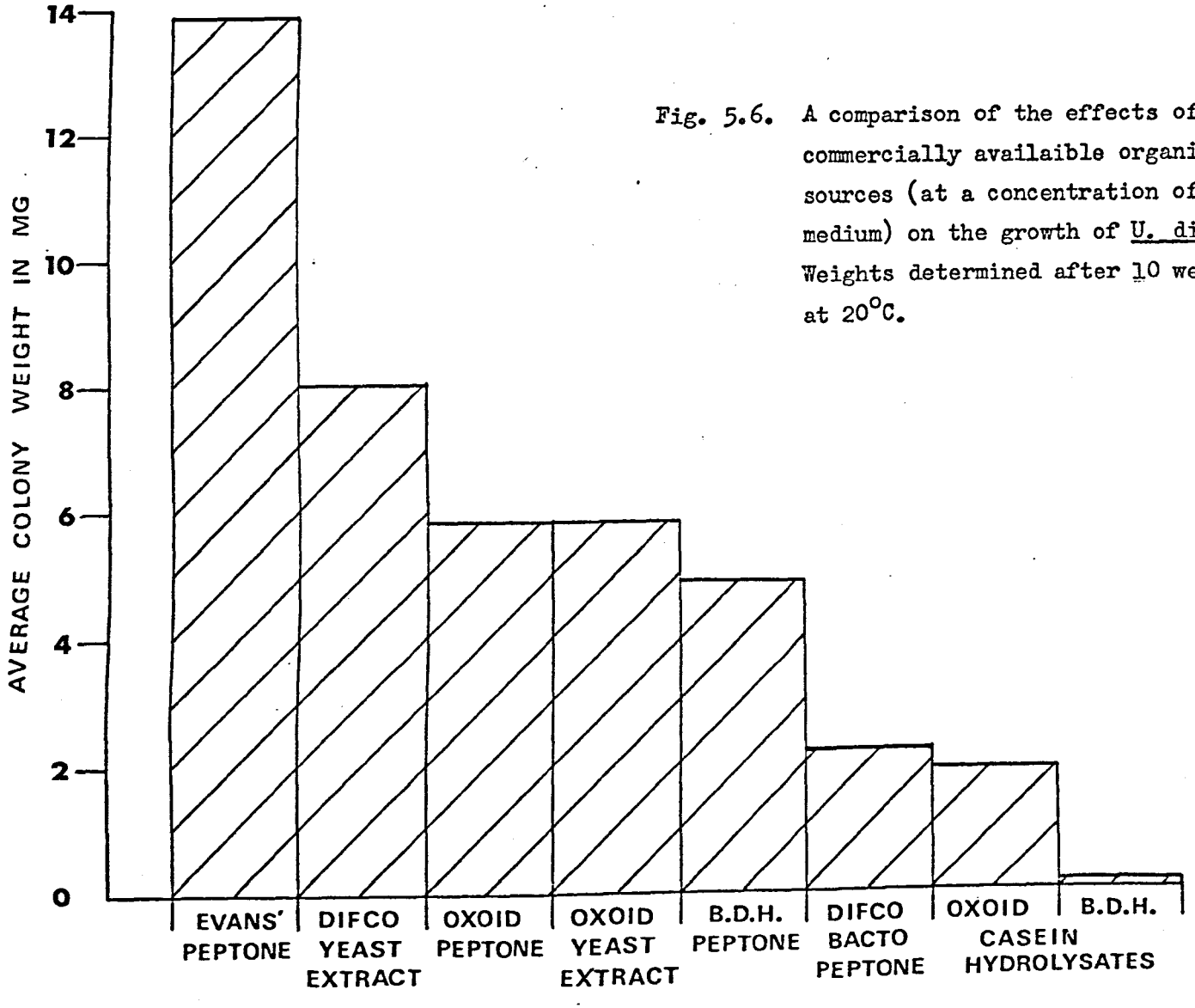
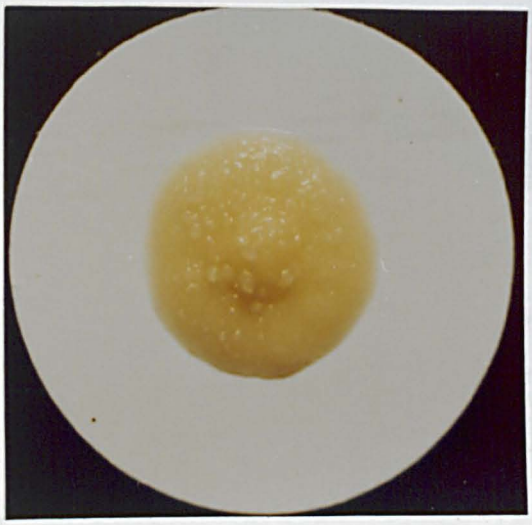


Fig. 5.6. A comparison of the effects of various commercially availaible organic nitrogen sources (at a concentration of 5 g/l of agar medium) on the growth of U. dianthi colonies. Weights determined after 10 weeks incubation at 20°C.



Pl. 5.2. Oxoid Yeast Extract medium.

Pl. 5.3. Difco Yeast Extract medium.

Pl. 5.4. Oxoid Peptone medium.

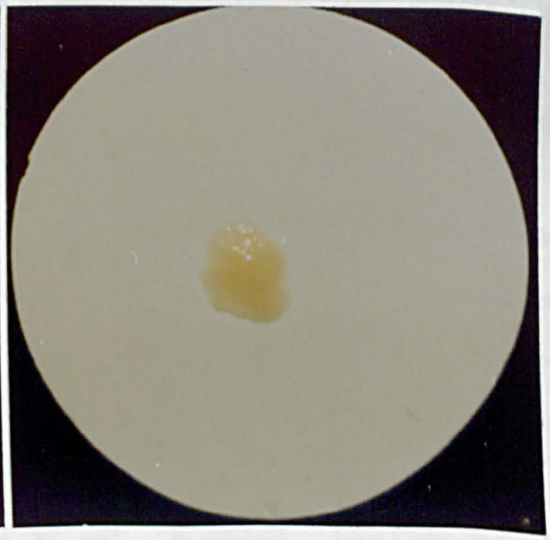
Pl. 5.5. Difco Bacto Peptone medium.

Plates 5.2-5. U. dianthi colonies after 10 weeks incubation at 20°C.

Pl. 5.6. B.D.H. Peptone medium.

Pl. 5.7. Evans' Peptone medium.

Pl. 5.8. Oxoid Casein Hydrolysate medium.



Pl. 5.9. B.D.H. Casein Hydrolysate medium.

Plates 5.6-9. U. dianthi colonies after 10 weeks incubation at 20°C.

medium agar contained a Millipore filtered sterile nitrogen source. From each of the three 'N' media ten plates were poured, and subcultures from 'A6' medium stocks (at 18°C) placed on sterile Millipore filter discs on the agar surface. After 10 weeks growth in a 18°C incubator, the weights of the colonies were found (see 5.22.2). The results were compared using Student's 't'-test.

Results. No statistically significant difference was found between the colony weights growing on the media containing the autoclaved Evans' Peptone. The dry weights of colonies on the autoclaved peptone media however was approximately twice the weight of those on the Millipore filtered peptone medium (Table 5.8). This difference was significant at the 1% level.

5.33.4 The effect on growth of individual amino acids.

Experimental procedure. To find if a single amino acid (as the sole nitrogen source) could support axenic growths, various agar media were prepared containing 0.2 g of an amino acid, 3.6 g of Difco Czapek Dox Broth, and 0.5 g of Difco Bacto Agar per 100 ml of distilled water.

Control media were prepared, one containing 0.2 g of Difco Yeast Extract, another containing o.2 g of ammonium sulphate, and another containing 0.2 g of ammonium nitrate in place of amino acids.

The amino acids used were alanine, aneurine, arginine, asparagine, aspartic acid, glutamic acid, glutathione (oxidised), glycine, lysine, methionine, nicotinic acid, phenylalanine, proline, serine, and tryptophan. Media pH's were adjusted with 0.1 N-HC1 and 0.01N-NaOH. After autoclaving, the pH values of all media lay in the range pH 5.8-6.0.

Three plates were poured per amino acid and control media.

TABLE 5.8. The weight of U. dianthi colonies grown on agar media containing Millipore sterilised and autoclaved Evans' Peptone (10 weeks incubation at 18°C).

	Evans' Peptone		
	Millipore Filtered	5 min Autoclaving	20 min Autoclaving
Colony Weights in mg	10.8	15.5	23.4
	10.5	24.2	25.6
	9.7	27.7	23.7
	9.1	25.2	20.9
	11.6	21.2	28.5
	14.2	28.2	23.8
	10.7	25.1	21.1
	7.8	35.3	17.9
	6.8	26.3	22.8
	10.9	21.3	20.1
Averages	10.2	25.0	22.8

All were inoculated with subcultures from 'A6' medium stocks (18°C) and these were incubated for several weeks at 18°C.

Results. The subcultures did not establish themselves on most of the plates. However, the subcultures on the yeast extract controls grew and there was a little growth on the plates containing methionine.

5.34 Carbohydrates and growth.

5.34.1 Sucrose concentration and growth.

Experimental procedure. Media were prepared with a range of sucrose concentrations from 0.25 g to 100 g per litre. The basis of these media was 2 g Difco Yeast Extract and 10 g Difco Bacto Agar per litre of Czapek Dox mineral solution. A control containing no sucrose were also prepared. All media were in the range of pH values 5.9-6.0 after autoclaving, and six plates were poured for each concentration of sucrose. Subcultures from 'A6' medium stocks (at 18°C) were transferred to Millipore filter discs on the agar surface. After an incubation period of 10 weeks at 20°C, the colony weights were determined (see 5.22.2).

Results. The average weight of the colonies increased as the sucrose concentrations in the medium increased. A little growth occurred on the control medium containing no sucrose (Table 5.9, Fig. 5.7).

5.34.2 Growth on various carbohydrate sources.

Experimental procedure. Media were prepared containing 30 g of various carbohydrates per litre. The basis of the media was 2 g Evans' Peptone, 2 g Difco Yeast Extract, and 10 g Difco Bacto Agar per litre of Czapek Dox mineral solution. The B.D.H. carbohydrates used were :-

1) D(-) Glucose

2) D(-) Fructose

TABLE 5.9. The weight of U. dianthi colonies grown on agar media containing different concentrations of sucrose (10 weeks incubation at 20°C).

Sucrose Concs.	Weight of Colonies in mg	Averages
0 g/1	0.0, 0.2, 0.2, 0.1, 0.1, 0.0,	0.1
0.25 g/1	1.0, 1.0, 1.5, 1.7, 1.3, 1.2,	1.3
0.50 g/1	1.6, 1.2, 1.7, 1.4, 1.3, 1.6,	1.4
1.00 g/1	2.3, 2.9, 1.8, 2.4, 2.4, 2.2,	2.3
10 g/1	4.3, 4.6, 4.1, 3.9, 4.2, 3.6,	4.1
20 g/1	3.9, 4.3, 4.6, 3.8, 4.7, 4.6,	4.3
30 g/1	5.2, 4.6, 5.3, 4.3, 4.0, 4.4,	4.6
40 g/1	5.1, 5.3, 4.5, 4.5, 4.9, 4.7,	4.8
50 g/1	5.9, 4.5, 5.7, 5.1, 5.6, 4.0,	5.2
60 g/1	4.2, 4.6, 4.1, 5.2, 5.3, 5.6,	4.8
70 g/1	7.3, 5.7, 6.6, 5.4, 3.6, 6.0,	5.9
80 g/1	6.5, 4.6, 6.4, 7.1, 6.2, 6.4,	6.2
90 g/1	4.9, 5.7, 6.2, 7.7, 5.4, 6.5,	6.2
100 g/1	7.4, 7.4, 6.3, 6.4, 5.4, 5.9,	6.5

TABLE 5.10. The weight of U. dianthi colonies grown on agar media containing different carbohydrates (10 weeks incubation at 20°C).

Carbohydrate	Weight of Colonies in mg	Averages
Glucose	8.4, 7.6, 7.8, 10.3, 8.5, 12.9,	9.3
Fructose	11.4, 8.3, 11.4, 9.1, 8.4, 7.8,	9.4
Sucrose	18.1, 18.7, 11.0, 13.6, 20.5, 9.3,	15.2
Maltose	10.3, 9.9, 9.0, 8.7, 5.7, 6.7,	8.4
Mannose	7.3, 7.1, 7.4, 5.5, 7.1, 6.5,	6.8
Mannitol	13.4, 13.9, 15.5, 14.0, 14.4, 8.8,	13.3

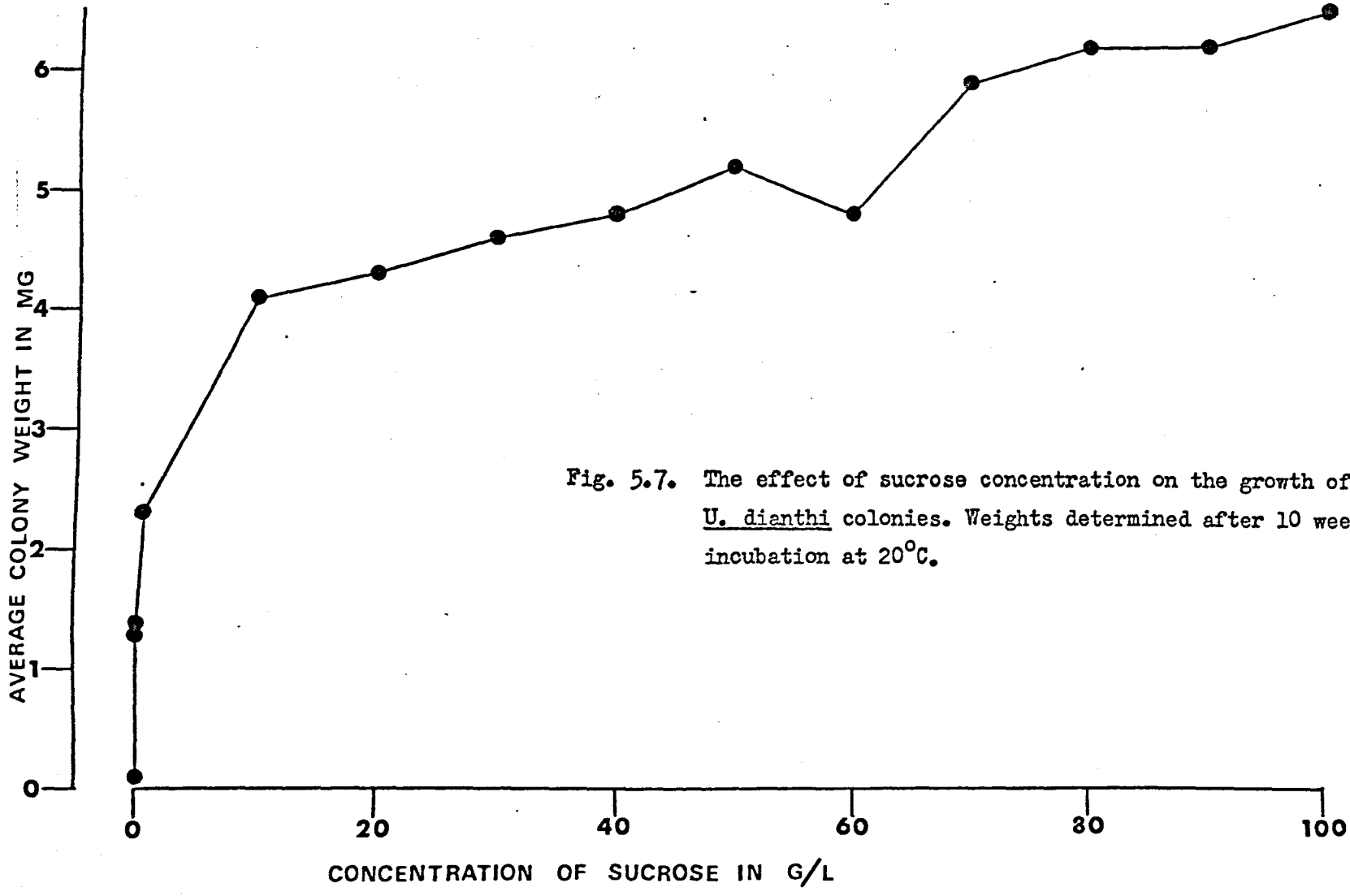


Fig. 5.7. The effect of sucrose concentration on the growth of U. dianthi colonies. Weights determined after 10 weeks incubation at 20°C.

3) Sucrose

4) Maltose

5) Lactose

6) D(+) Mannose

7) Mannitol

8) Erythritol

9) B(+) Galactose

10) Starch

After autoclaving for 20 min at 110°C, six plates were poured from each carbohydrate medium, all of which were of pH 6.0. Subcultures from 'A6' medium stocks (at 20°C) were placed on Millipore filter discs resting on the agar surface. After an incubation period of 10 weeks at 20°C, the colonies were weighed (see 5.22.2), and their weights compared statistically using Student's 't'-test.

Results. Colonies did not establish themselves on the media containing lactose, erythritol, galactose and starch, but growth occurred on the glucose, fructose, sucrose, maltose, mannose and mannitol media (Table 5.10). No significant difference was found between the weights of the glucose, fructose and maltose colonies, and between the glucose, maltose and mannose colonies. The fructose and mannose colonies were statistically different at the 1% level of significance. There was also a significant difference at the 5% level between the glucose, fructose, maltose and mannose colony weight and the greater weight of the sucrose and mannitol colonies. The weights of the mannose and mannitol colonies were different at the 0.1% level of significance. There was no significant difference between the sucrose and mannitol colony weights.

5.4 DISCUSSION

The results showed that the pH limits within which axenic growth was possible were between pH 5.3-5.5 and pH 6.8-7.0. It

was also found that the optimum growth occurred on an agar medium of initial pH 6.0, confirming what had been indicated earlier (see Chapter 4.31.2). Drift of the medium pH was known to occur however, as after the pH 5.8-6.2 experiment (see 5.32.1), the pH values of the media were found to be lower than they were originally by approximately pH 0.2-0.3. This discovery initiated research into the possible relationship between pH drift and the cessation of growth (see Chapter 6.37). As no experiments were performed successfully using buffered media, the accurate optimum pH value is not known, but is believed to be in the region of pH 5.9-6.0.

The pH limits for the growth of U. dianthi are much narrower than for most fungi. This is probably because as an ecologically obligate parasite, it's metabolism is adapted to the normal pH values and limits of its host's tissues.

The pH limits of other rusts in axenic culture are not known. Singleton & Young (1968) however, found that the optimum growth of P. recondita f. sp. tritici was on media of pH 6.0-6.2 and the pH value favoured by workers using P. graminis f. sp. tritici has been pH 6.4 (Williams et al., 1966; Williams et al., 1967; Bushnell, 1968; Coffey et al., 1969). Turel (1969a) worked with media of pH 5.7-6.2 when culturing M. lini, but Coffey et al., (1970), working with the same fungus, used media of pH 4.9-5.1. At media levels between pH 4.5 and 5.6, growth of M. lini mycelium on flax leaf tissue cultures was maximal (Turel & Ledingham, 1957). It seems therefore that the Puccinia species so far cultured have optimum pH values similar to that of U. dianthi. M. lini however, may have a significantly lower optimum pH value.

The highest growth rates of U. dianthi were recorded at the temperature of 18°C. This again confirmed observations

(see Chapter 4.31.2), and was similar to the incubation temperature of 17°C used to culture P. recondita (Singleton & Young, 1968) and P. graminis (Williams et al., 1966). Turel (1969b) found that M. lini grew better at 16°C than at 17°C, and that cultures were seriously damaged after being kept at 24°C for 30 to 45 min before transfer. This contrasted with U. dianthi, which was less temperature sensitive and could grow at temperatures up to 28-30°C.

From the experiments presented, it appears that for maximum growth, the nitrogen source contentration in the medium should be around 10 g/1. Early axenic rust workers used the relatively low concentrations of 1-2 g/1. Bushnell & Rajendren (1970) however found that between 7 and 10 g/1 of Difco Casein Hydrolysate was optimum for growth of P. graminis. It is possible that certain concentrations are suitable for saprophytic initiation, and others for its subsequent colonizing growth.

Difco Yeast Extract and Evans' Peptone were the nitrogen media used by early rust axenic culture workers to initiate and sustain the saprophytic development of P. graminis, P. recondita and M. lini. A comparison of the effects of various commercial nitrogen media on U. dianthi, showed that at the concentrations used (5 g/1), Evans' Peptone was by far the best growth promotor, and Difco Yeast Extract the second best. Casein hydrolysates seemed the least capable of supporting growth. This indicates that although saprophytic growth is possible on media containing amino acids as the sole nitrogen source (casein hydrolysates), other substances present in Evans' Peptone and Difco Yeast Extract (possibly certain vitamins) are necessary for good growth. Kuhl et al. (1970) found Evans' Peptone better for culturing P. graminis than any other peptone tried.

The medium with casein as the sole nitrogen source did

not support growth at all. This indicated that *in vitro*, *U. dianthi* is incapable of producing protein degrading enzymes in sufficient quantities to support itself. The significant increase in the growth promoting properties of Evans' Peptone when autoclaved was believed to be because of the breakdown by heat of various polypeptides to amino acids, thus increasing the amount of available nitrogen.

Kuhl *et al.*, (1971) found that when initiating axenic cultures of *P. graminis*, yeast extract and peptone could be replaced in the medium by aspartic acid and a sulphur containing amino acid, but not methionine. However, Coffey & Shaw (1972) found that alanine and methionine were necessary for the axenic growth of *M. lini*. The results of the amino acids and growth experiment presented here, suggests that methionine, a sulphur containing amino acid, may have a key role in the nitrogen metabolism of *U. dianthi*, as it was contained in the only amino acid medium on which growth occurred. *U. dianthi* was found incapable of utilising inorganic nitrogen salts.

Experiments showed that the growth of *U. dianthi* increased as the concentration of the sucrose in the medium rose to 100 g/1. A little growth also occurred on a control medium containing no sucrose, indicating that biochemical pathways existed in *U. dianthi in vitro* for converting nitrogen sources to carbohydrates. The limited amount of this growth can be explained by the low nitrogen content of the medium (2 g/1) and an insufficient rate of conversion for normal development.

Colonies of *U. dianthi* have also been grown on media containing up to and including 200 g of sucrose per litre. *U. dianthi in vitro* can therefore withstand osmotic pressures produced by 0.5871 M of sucrose or 17.31 atmospheres. This corresponds closely to an average osmotic pressure of 18.6

atmospheres found for U. dianthi haustoria (Thatcher, 1939). In vivo, the osmotic pressure of the fungus cell must be higher than that of the plant cell in order for the haustoria to obtain water. Thatcher (1939) found the average osmotic pressures of Dianthus leaf bases to be 11.2 atmospheres.

Kuhl et al. (1971) found that fructose, glucose, mannitol and mannose could be substituted for sucrose in the P. graminis axenic initiation medium, and Coffey & Shaw (1972) showed that saprophytic growths of M. lini could utilize fructose, glucose, maltose, mannitol, mannose, raffinose, ribitol and sorbitol in addition to sucrose.

Research presented, indicated that fructose, glucose, maltose, mannitol, mannose and sucrose could also support axenic growths of U. dianthi. Galactose, which was not suitable for the saprophytic initiation of P. graminis or M. lini was also found unsuitable for the U. dianthi cultures. In addition, it was found that erythritol, lactose and starch could not support growth.

The inability of U. dianthi to utilise lactase and starch indicated that lactose (the enzyme required for the breakdown of lactase to glucose and galactose) and β-amylase (starch reducing enzyme) were not present in vitro. If similar conditions hold in vivo, it would mean that the process of plant starch reduction to sucrose would be carried out by enzymes of host origin. As U. dianthi was capable of utilising sucrose, it must have possessed the enzymes (invertase or sucrose phosphorylase) required to breakdown to glucose and fructose. The hydrolysing enzyme maltase must also have been present for the breakdown of maltose to glucose.

At the carbohydrate concentrations used (30 g/1), sucrose and mannitol promoted the maximum growth. As sucrose accumulates

as a product of photosynthesis, and appears to be the major soluble reserve carbohydrate in higher plants (Davies, Giovanelli & Ap Rees, 1964) one would expect plant pathogens to utilise this readily.

Most fungi appear to utilise sugars with greater facility than the corresponding sugar alcohols (Lilley & Barnett, 1951), so it is surprising that mannitol encouraged the growth of U. dianthi far more than mannose. Mannitol however, is known to be formed in fungi as a reserve carbohydrate (Hawker, 1950) and there is evidence of its formation from translocated sucrose in Ustilago nuda infected wheat ears (Gaunt & Manners, 1971).

The differences in comparable colony weights in the various experiments performed in this chapter are due to the gradual adaption of U. dianthi to its saprophytic environment (see Chapter 6.38).

CHAPTER 6. STUDIES ON THE DEVELOPMENT OF SAPROPHYTIC GROWTHS OF U. DIANTHI

6.1 INTRODUCTION

With most U. dianthi cultures, colonization of the saprophytic hyphae across an agar medium usually ceased within 10-18 weeks. During the cessation period, the colonizing process was observed to slow gradually, and the marginal hyphae to resemble those usually associated with the interior of the colony. At the time of cessation, all hyphae appeared morphologicallly similar and colonies could be easily fragmented. The relationship of colony growth and time were determined and also the sequence of hyphal growth and differentiation.

Discolouration of colonies of P. graminis f. sp. tritici race 126-ANZ-6, 7 and surrounding medium was observed by Wong & Willetts (1970) to be closely associated with the loss of viability of the fungus. They found that the definite browning of the mycelium developed 14 days after seeding and coincided with growth cessation. Thin layer chromatographic studies showed that at least one phenolic compound present in pigmented colonies was missing in non-pigmented ones. A toxic oxidised polyphenol was suggested as the compound responsible for the pigment.

Growth cessation in U. dianthi occurred long before the greater part of the agar surface was colonised and this suggested that either something was inhibiting growth, or that nutrients were being exhausted in the medium (the colonies acting as 'sinks'). The loss of viability of U. dianthi in vitro was investigated.

6.2 MATERIALS AND METHODS.

6.21 Agar media and pH.

Media were prepared and pH values recorded as previously

described (see Chapter 4.23).

6.22 Stock cultures

All U. dianthi stock cultures were maintained on either 'A6' or 'N' medium plates (see Chapter 5.21.2).

6.23 Colony diameters and weights.

Colony diameters were measured as described previously (see Chapter 4.21.3), and colony dry weights determined by the Millipore filter disc method (see Chapter 5.22.2).

6.24 Preparation of a known volume of agar medium per plate.

The agar medium was first autoclaved at 120°C for 1 min, and then 40 ml measured into large boiling tubes using an Everett agar dispenser. Metal caps were placed on the boiling tubes and the agar was reautoclaved at 120°C for 15 min. The sterile 40 ml volumes of liquid medium were then poured into plastic Petri dishes and allowed to cool.

6.3 RESULTS.

6.31 The growth of U. dianthi colonies with time.

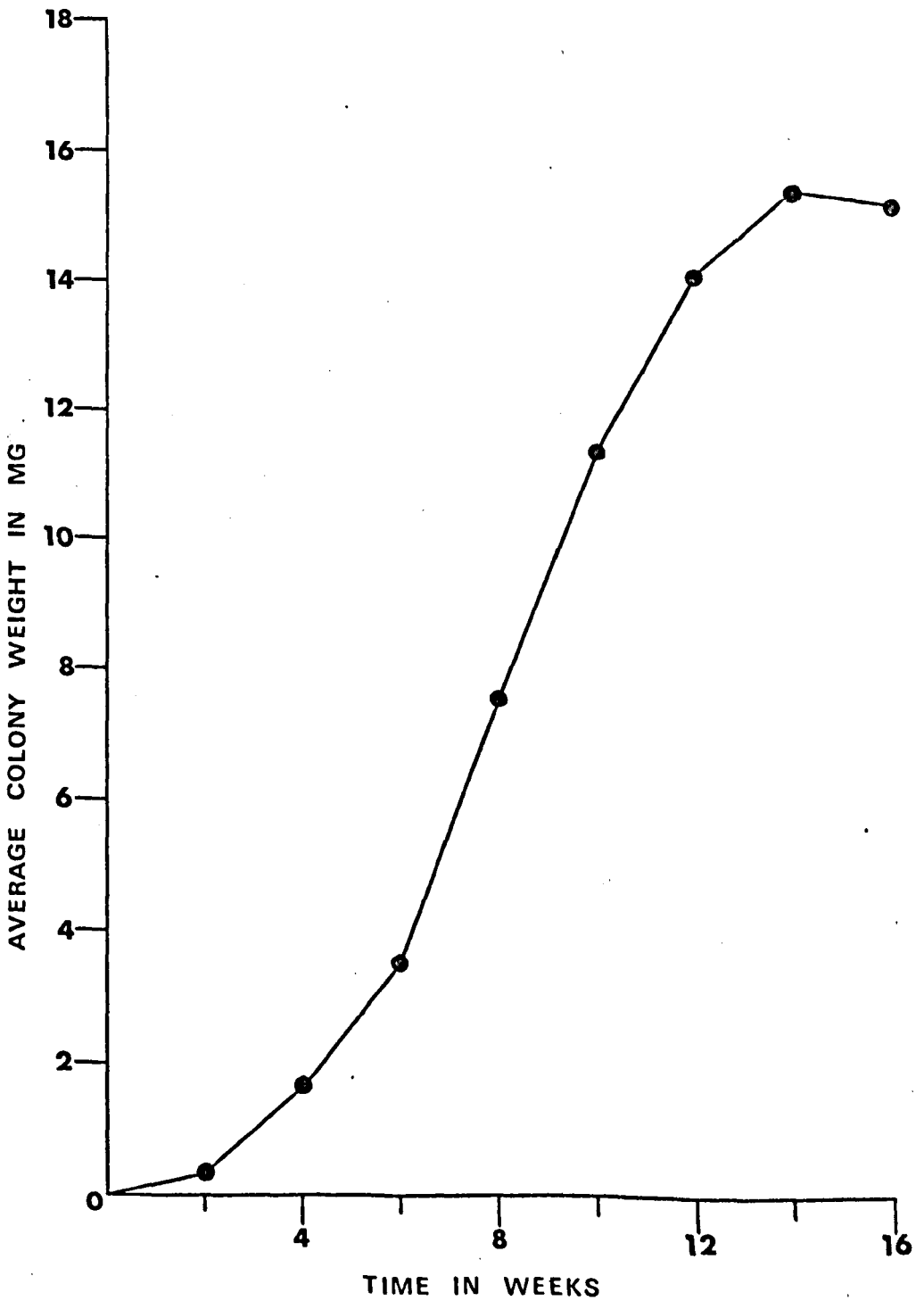
Experimental procedure. 'A' medium agars (pH 6.0) were prepared and seventy plates poured. Subcultures from 'A6' medium stocks (20°C) were transfered to Millipore filters on these plates. The plates were then placed in a 20°C incubator and eight removed at random after 2,4,6,8,10,12,14 and 16 weeks. After removal, the dry weights of the colonies were determined and statistical comparisons made using the Student's 't'-test.

Results. From the average colony weights (Table 6.1), a graph was plotted which gave a typical sigmoid growth curve (Fig. 6.1). The average weight of the colonies increased markedly between the sixth and twelth weeks, growth ceasing after about 14 weeks. The dry weight of colonies between 2,4,6,8, 10 and 12 weeks were found to be statistically different from each other at at least the 5% level of significance. Average colony

TABLE 6.1 The effect of incubation time at 20°C on the dry weight of U. dianthi colonies.

Incubation Time in	Colony Weights in mg	Averages
2	0.6, 0.2, 0.2, 0.3, 0.3, 0.2, 0.7, 0.3	0.35
4.	1.1, 1.8, 1.7, 1.9, 2.0, 1.6, 1.6, 1.8	1.69
6	3.9, 4.2, 3.4, 4.3, 3.1, 3.4, 2.9, 2.9	3.51
8	6.0, 6.0, 5.7, 8.8, 9.5, 8.7, 8.5, 7.2	7.55
10	9.8, 12.4, 11.2, 9.2, 10.2, 14.8, 11.7, 11.4,	11.34
12	15.8, 13.8, 12.7, 16.2, 13.1, 16.6, 10.2, 14.2	14.08
14	14.0, 16.0, 15.1, 15.6, 16.6, 14.9, 15.1, 15.7	15.38
16	14.7, 17.8, 17.5, 14.8, 15.0, 12.8, 17.5, 11.2	15.16

Fig. 6.1. The relationship of growth, as expressed by U. dianthi colony weight, and time.



weights between 12 and 16 weeks were not found to be significantly different.

6.32 Hyphal differentiation.

Experimental procedure. Photomicrographs were taken under phase contrast of marginal hyphae of 6 week old U. dianthi stock cultures growing on 'N' medium agar. From them a sequence of hyphal differentiation was established.

Results. The branching of U. dianthi hyphae in vitro was monopodial, the apex of the leading hypha keeping pace in growth with the most active of the lateral branches. This was in agreement with the rust in vivo observations of Arthur (1929).

Septa were formed at intervals along the main hyphae, and these were invariably convex in shape as a result of the cytoplasmic flow pressure (Pl. 6.1). At some septa, bulges became noticeable in the hyphal cell immediately below them (Pl. 6.2). These gave rise to lateral side growths which in their early stages resembled clamp connexions (Pl. 4.2). Occasionally two side branches would form (Pl. 6.3). The reason for the initiation of the branching is not known, but it may be a result of nuclear division stimulating cell development. Further growth resulted in knob-like branches (Pl. 6.4), and subsequently led to the formation of long lateral branches (Pl. 6.5), which were occasionally stimulated to divided dichotomously (Pls. 6.6, 6.7).

Hyphal apices growing a long way behind the main hyphae were observed to become slighty disorganised (Pl. 6.8), and produce many knob-like projections (Pls. 6.9, 6.10). These became more pronounced (Pl. 6.11) and eventually resembled those usually associated with the colony interior (Fig. 4.2).

6.33 The effect of light on hyphal growth.

Experimental procedure. Colonies growing on 'A' medium plates (pH 6.0, 18°C) were placed in lighted incubators (50

Plate 6.1. X 1404.

Plate 6.2. X 1664.

Plate 6.3. X 1404.

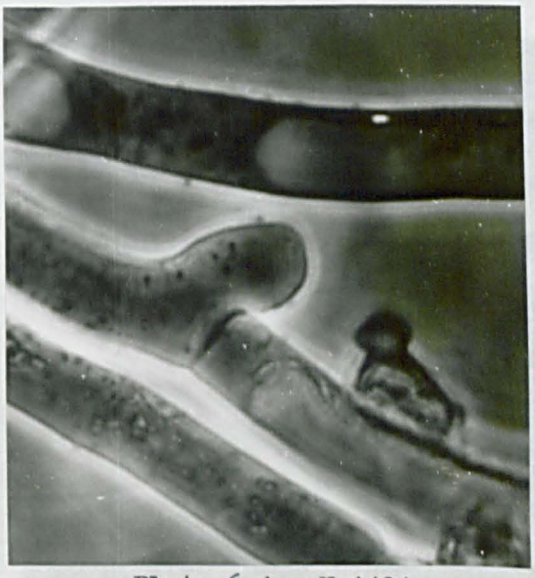


Plate 6.4. X 1404.

Plate 6.5. X 676.

Plates 6.1-11. U. dianthi hyphae growing on the surface of 'N' medium agar (pH 6.0) at 18°C.

Plate 6.6. X 1404.

Plate 6.7. X 884.

Plate 6.8. X 1664.

Plate 6.9. X 1664.

Plate 6.10. X 1664.

Plate 6.11. X 1664.

lumens ft.^{2}) at 18^{o}C. Some colonies were given continuous light, others 12 h light, 12 h dark. After 4 days, the hyphae were examined, photomicrographs taken, and the plates replaced in the incubators.

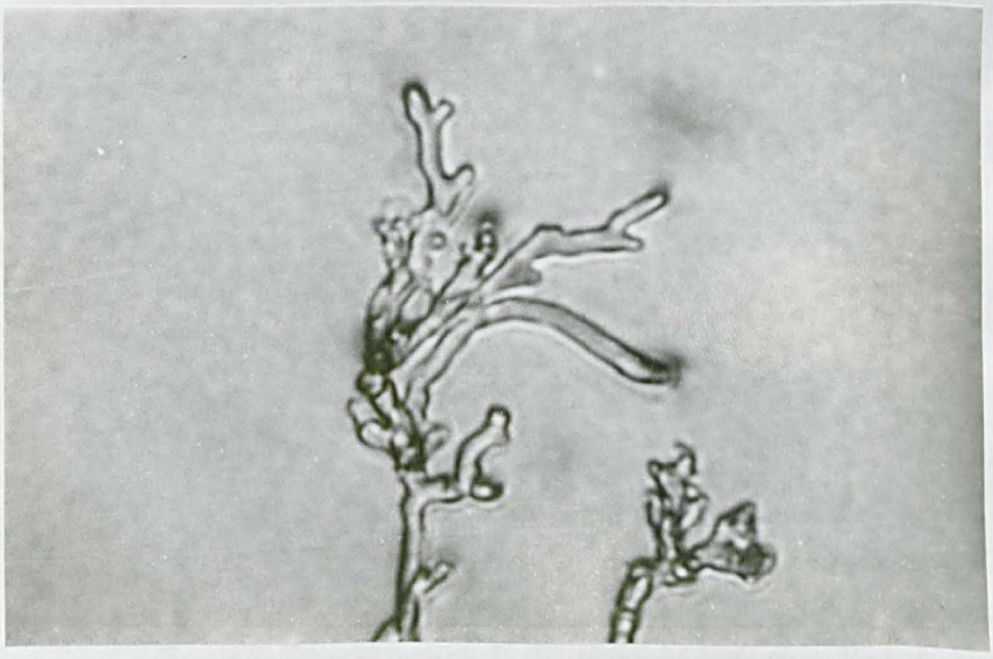
Results. After 4 days the colonies in continuous light showed a high degree of hyphal apical branching and distortion (Pls. 6.12, 6.13). The apices of hyphae from colonies in 12 h light and 12 h dark were also distorted, but not to such a great extent. The distortion resembled that found in hyphal apices growing behind the colonising hyphal margin (Pls. 6.9, 6.10). Occasionally spore-like cells were formed.

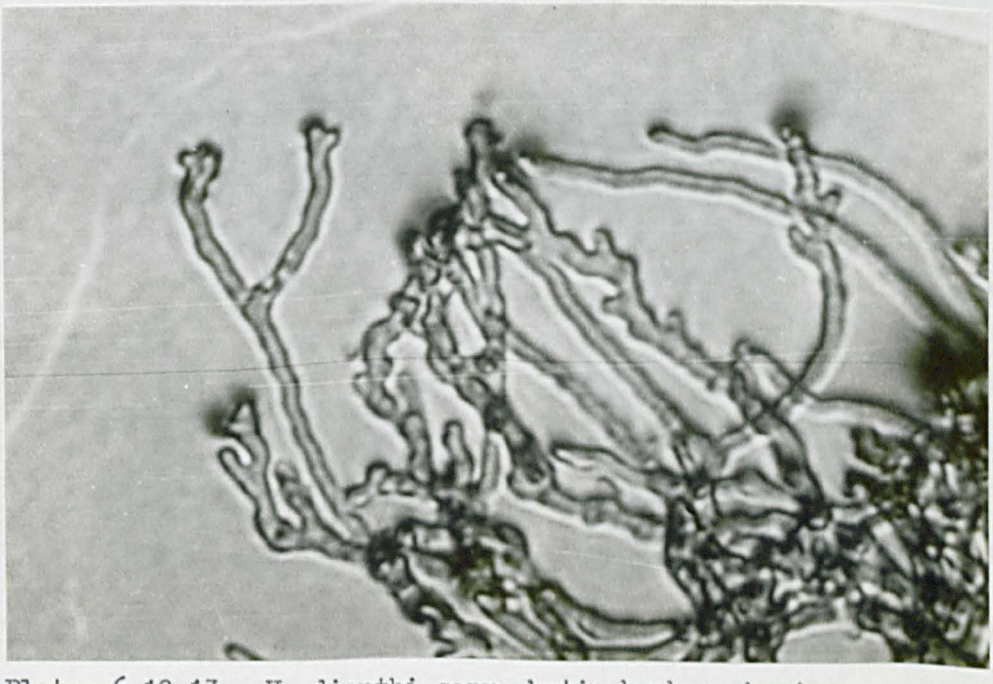
After prolonged exposure to the light source (between 1 and 2 weeks), the hyphae died.

6.34 The effect of the number of colonies per plate on colony weight.

Experimental procedure. Exactly 40 ml volumes of 'A' medium agar (pH 6.0) were poured into forty-one Petri dishes (see 6.24). One Millipore filter disc (25 mm in diameter) was placed on the centre of the agar surface on twenty of these plates. Two Millipore filters were positioned on the agar surfaces of ten plates, three on six plates, and four on five plates. All filters were arranged so as to be equidistant from each other. Subcultures from 'A6' medium stocks (20^{o}C) were placed on the filters, and the plates were incubated for 15 weeks at 20^{o}C. After this time, when all growth had ceased, the dry weights of the colonies were found.

Results. The weights of the single colonies were much greater than those of the double colonies, which were in turn greater than the colony weights from the three-colony plates. The colonies on the four-colony plates were the lightest of all (Table 6.2). This was evident from their compartive sizes





Plates 6.12-13. U. dianthi saprophytic hyphae showing branching and distortion after 4 days in continuous light. X 800.

TABLE 6.2. The relationship between colony weight and the number of colonies per plate.

	One Colony per Plate	Two Colonies per Plate	Three Colonies per Plate	Four Colonies per Plate
Colony Weights in mg	14.1	9.3	4.7	3.9
	13.7	7.3	4.9	2.2
	12.7	6.3	4.3	2.1
	13.2	9.5	4.3	3.9
	12.1	7.4	3.9	2.6
	14.9	5.8	4.0	2.3
	12.5	4.6	5.8	2.1
	14.2	6.5	5.6	2.5
	14.8	7.7	4.0	2.8
	11.9	7.4	3.5	3.3
	13.6	6.6	4.1	3.5
	10.8	6.8	4.1	3.3
	12.0	7.4	4.0	2.5
	12.5	12.1	4.5	3.1
	14.6	6.2	4.0	2.9
	10.7	7.4	4.8	2.8
	14.2	6.8	4.8	3.7
	11.2	5.3	4.8	2.5
	12.4	7.3		2.8
	11.3	8.5		3.2
Average Colony Wt.	12.87	7.31	4.45	2.90
Average Wt. of Colonies/ Plate	12.87	14.62	13.35	11.60

before weighing (Pl. 6.14). The total weights of colonies per plate were however similar. A log graph which was plotted showed a direct relationship between colony number per plate and individual colony weight (Fig. 6.2).

From Table 2, the average of the average total weight of colonies per plate was found to be 13.11 mg. From this, it was calculated that 10 ml of 'A' medium (pH 6.0) at the time of the experiment could support approximately 3.37 mg of saprophytic U. dianthi hyphae.

Conclusions. The results indicated that growth ceased when either 1) the colonies exhausted essential growth nutrients in the medium, or when 2) growth inhibiting metabolites produced by the colonies built up to a critical level.

6.35 The effect of a plate containing a mature colony on further subculturing.

Experimental procedure. Actively colonizing marginal hyphae were subcultured from 'A6' medium stocks (18°C) to three 'A' medium plates (pH 6.0). The subcultures were transferred directly to the agar surface and positioned towards the side of the plate. Three 'A' medium plates were not inoculated (controls). All six plates were incubated at 18°C until growth of the colonies on the inoculated plates ceased. This occurred after approximately 15 weeks. Subcultures again from 'A' medium stocks (18°C) were then used to inoculate all the plates, including the controls. The subcultured fragments were placed on the agar at the opposite side of the plate from the original inoculation.

Results. The second subcultures failed to form colonies on the three plates that had supported a previous subculture, but formed large colonies on the three controls (Pl. 6.15).

Conclusions. The second subcultures failed to grow on

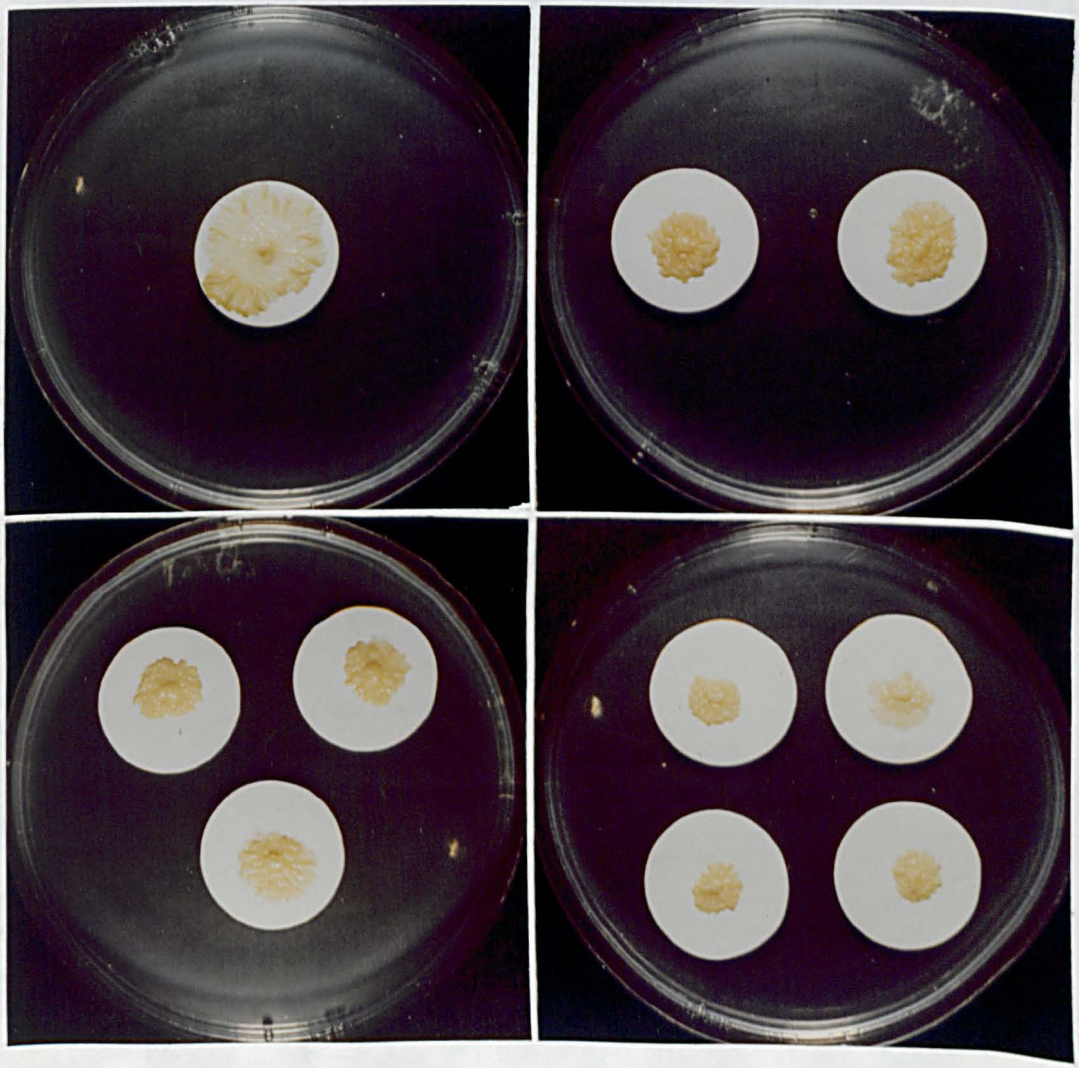


Plate 6.14. U. dianthi colonies after 15 weeks incubation at 20°C on 'A' medium plates. As the number of colonies per plate increases, the size of the colonies decreases.

Fig. 6.2. Graph of the log average U. dianthi colony weight against the number of colonies per plate. Weights determined after 15 weeks incubation at 20°C.

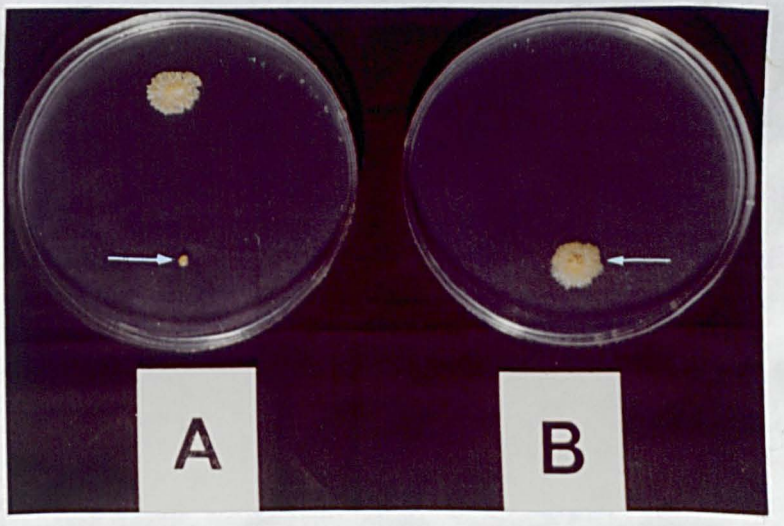


Plate 6.15. A) U. dianthi subculture (arrow) after 15 weeks incubation at 18°C on an 'A' medium plate which supported the growth of a mature U. dianthi colony.
B) U. dianthi subculture (arrow) on an 'A' medium control plate after 15 weeks incubation at 18°C.

Plate 6.16. Pale orange U. dianthi colony after 15 weeks incubation on an 'A' medium plate at 20°C.

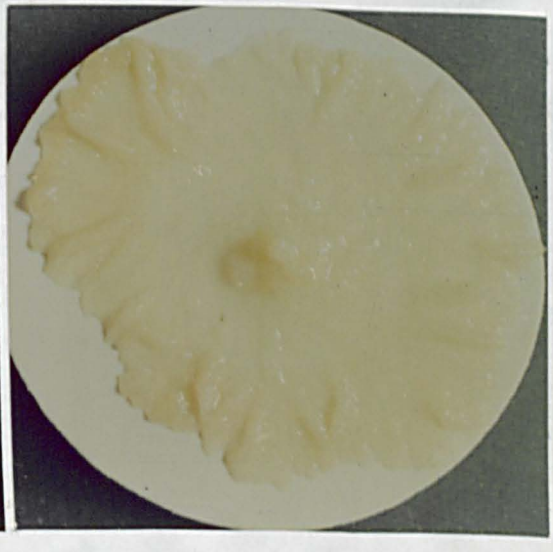


Plate 6.17. Yellow U. dianthi colony after 15 weeks incubation on an 'A' medium plate at 20°C.

the plates that had previously supported a colony because the old colonies had either exhausted essential growth nutrients, or had produced an inhibiting metabolite which prevented further growth on the medium.

6.36 Pigmentation and colony growth.

The cessation of growth of U. dianthi could not be associated with such a definite colour change of the mycelium as was observed by Wong & Willetts (1970) with P. graminis f. sp. tritici. As reported (see Chapter 4.31.2), the colour of U. dianthi colonies usually changed from white through yellow to pale orange (Pl. 6.16). The orange colour deepened slighty after growth had stopped. When some old U. dianthi colonies, cultured on Millipore filters, were removed from their plates, especially those of high carbohydrate content, slight cloudy white colourations were observed in the agar directly beneath the colonies. On one plate, a pale orange colour was noticed in the agar.

Occasionally colonies remained yellow in colour throughout their growth (Pl. 6.17). Other colonies developed a very dark pigmentation, especially in their centre (Pl. 6.18). This was true of all colonies grown on media with mannitol as the carbohydrates base (Pl. 6.19). Subcultures on media containing tryptophan as the sole nitrogen source (see Chapter 5.33.4) developed a dark brown colour (Pl. 6.20). This suggested that tryptophan encouraged, or was involved in the development of the pigment.

6.37 pH drift in media supporting U. dianthi colonies.

Although most fungi can grow on media with very low pH values, it was found in initiation and subculturing experiments (see Chapters 4.31, 5.31) that U. dianthi would not develop or grow on media below pH 5.3-5.4. The agar in about forty plates

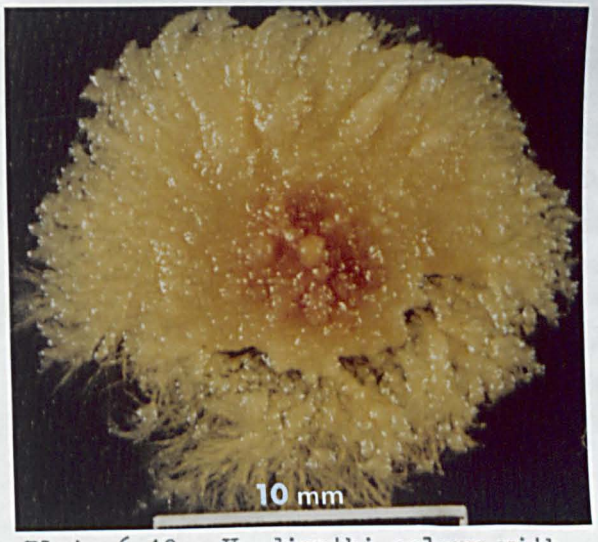


Plate 6.18. U. dianthi colony with a central dark orange pigmentation zone (after 15 weeks incubation at 20°C on an 'A' medium plate).

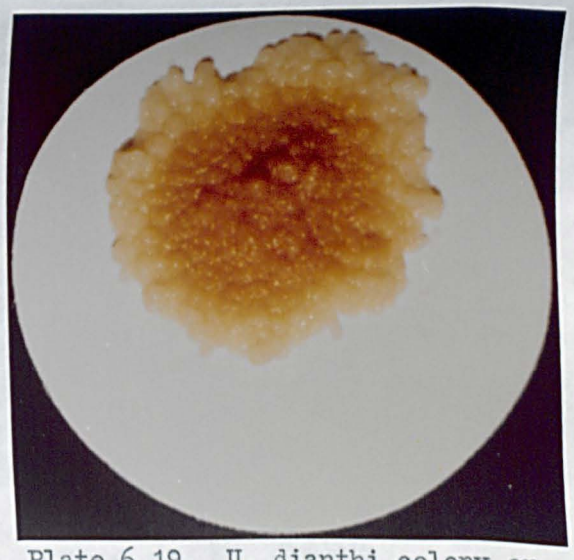


Plate 6.19. U. dianthi colony on a medium containing mannitol as the sole carbohydrate source (after 10 weeks incubation at 20°C).

Plate 6.20. U. dianthi sub-culture on a medium containing tryptophan as the sole nitrogen source.

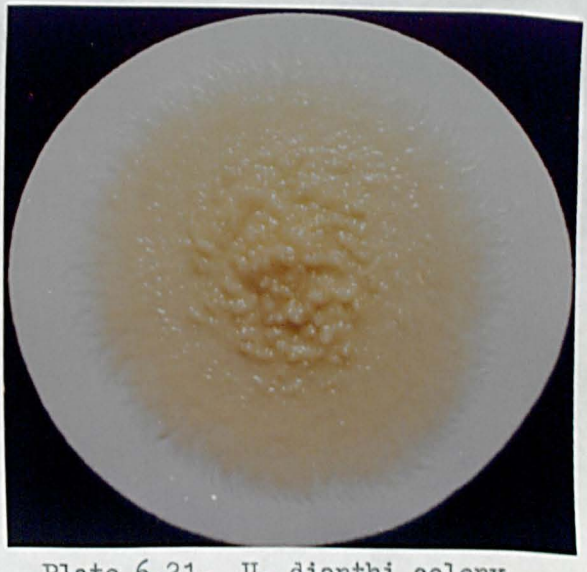


Plate 6.21. U. dianthi colony 45.5 mm in diameter and weighing 27.6 mg (after 15 weeks incubation at 18°C on a 'N' medium plate).

on which U. dianthi colonies had grown to maturity and had subsequently died, were individually melted and their pH's tested. In every case the pH of the media was found to be between pH 5.30 and pH 5.35. This suggested, as the original pH's of the agars varied from pH 5.8 to pH 6.4, that U. dianthi produced metabolities in vitro that lowered the pH of the media to an inhibitory level. The pH-lowering metabolites were thought to be organic acids produced from carybohydrates in the medium.

To test the pH-inhibiting theory, buffered 'N' media (pH 6.0) were prepared containing 0.01 M and 0.001 M solutions of phosphate buffer. It was hoped that these media would buffer the pH changes, and enable the U. dianthi colony to spread across the whole of the plate. The subcultures failed to grow on these agars however, possibly because of the fungitoxic action of high phosphate concentrations, but more likely because of the genetic failure of the saprophytic hyphae (see 6.38).

6.38 Adaption of U. dianthi to the medium.

As the stock cultures were repeatedly subcultured, it became noticeable that colonies were growing faster for longer periods and were subsequently larger. In Experiment 6.34 it was found that the average colony weight for four colonies per plate was 2.90 mg. When this part of the experiment was repeated 6 months later, the average weight was found to have increased to 6.74 mg.

Colonies repeatedly subcultured as 'N' medium stocks became very adapted to their saprophytic environment. The largest colony grown on a Millipore filter disc (Code HAWP 04700, 47 mm in diameter, 0.45 μm pore diameter) was 45.5 mm in diameter and weighed 27.6 mg (Pl. 6.21). The pH of the agar supporting these colonies was found to be 5.15-5.20 after growth had stopped. It was thought that the adaption may have been due to an ability

of these strains of U. dianthi to tolerate a lower pH, and also perhaps to produce less organic acids and consequently slow the pH drift.

After several transfers, subcultures from these well adapted colonies failed to grow when transferred to new 'N' medium stock plates. It was believed to be caused by genetic failure of the saprophytic hyphae, probably due to the repeated selection of certain fast growing strains over the months.

6.4 DISCUSSION.

The development of U. dianthi cultures followed a typical growth curve pattern, with growth ceasing long before the medium was fully colonised. Experiments performed showed that growth ceased either because of the exhaustion of an essential nutrient, or because of the production of a toxic metabolite. Because it was found that all media on which early colonies had ceased growing had a pH of 5.30-5.35 (the limit of U. dianthi tolerance in subculturing experiments), it was considered that growth was inhibited by a build-up of the hydrogen ion concentration in the media.

As many fungi synthesize organic acids, especially in environments containing excess carbohydrate, and lower the pH of their media (Lilley & Barnett, 1951), it was thought likely that acids were responsible for the pH drift of the medium. The slight cloudy whiteness observed in some agars (especially those of high carbohydrate content), after the removal of U. dianthi colonies may have been caused by the precipitation of salts of organic acids (e.g. calcium oxalate).

It was believed that the 'N' medium stock culture colonies developed an ability to tolerate lower pH values, and perhaps to produce less organic acids, through selective subculturing over many months. The largest, 'healthiest', appearing stock

colonies were invariably chosen to initiate new series of stock subcultures. The eventual loss of viability of the fungus was also thought to be due to the selection of these fast growing but genetically weak strains.

The hydrogen ion concentration of the medium is known to alter the pH of the fungal cell and effect various cell processes (Lilley & Barnett,1951). This was thought to be a possible reason for the differentiation of U. dianthi hyphae from the filamentous colonizing type of growth, to the knobbed,branched appearing hyphae, and finally to the production of spore-like cells. Wong & Willetts (1970),working with P. graminis f. sp. tritici, associated growth cessation, and the formation of numerous short swollen branches and thick walled, septate, enlarged hyphal tips, with the onset of pigmentation. In the course of their research however, they found that although polyphenoloxidase inhibitors (polyphenoloxidases were thought responsible for the formation of the pigment, believed to be an oxidised polyphenol) reduced pigmentation when added to the medium, they did not prolong growth. This seemed to indicate that the pigment of the media was perhaps not responsible for growth cessation. No pH values of the media were taken by Wong & Willets, but it is unlikely that a substantial pH drift could occur during the 14 days of mycelial growth. If so, a lowering of the pH to an inhibitory level would not seem to be the cause of growth cessation. If the critical pH level of P. graminis was just below the initial pH value of the medium however, pH drift could still be responsible.

Similar hyphal differentiation processes seemed to occur to the hyphae on exposure to light. This too could be explained by upset metabolism. It was found with Blastocladiella emersonii that light stimulated the production of nucleic acids and other cellular reactions (Turian & Cantino, 1959; Cantino & Turian, 1961). The hyphal dichotomies and swelling of U. dianthi

following exposure to light were probably caused by disturbed metabolic processes which culminated in the death of the mycelium.

Fungitoxic 'staling substances' that are produced and accumulate when plant parasites are grown in culture have been said to accumulate in plant tissues when they are used as a substrate. Wood (1967) thought that resistance due to a change in tissue pH may be commoner than one would suppose from lack of examples. It has been suggested that resistance of some tobacco varieties to Pseudomonas tabaci depended on the development of an alkaline pH at the site of inoculation (Garber, 1961). A high degree of acidity in the cell sap may confer resistance against pathogens intolerant of an acid reaction. The increased susceptibility of older apple trees to rotting is associated with decreasing acidity (Hawker, 1950), and immunity of monocotyledonous plants to the cotton-rot fungus Phymatotrichum omnivorum has been attributed in part to the presence of acids in the cell sap (Ezekial & Fudge, 1938).

If the rust mycelium stimulates the production of carbohydrates, as is claimed by many workers (Hassebrauk & Kaul, 1957; Gerwitz & Durbin, 1960; Inman, 1962), it is possible that organic acids are produced during their respiration, and that these would lower the pH of the infected tissues. The pH value dropping below a certain critical level may well limit hyphal development and growth, and could be partly responsible for symptom expression and spore production.

The function of pigments in fungi are not well understood. The production of the orange pigmentation found in U. dianthi may be influenced by hydrogen ion concentration, as the pigment usually deepened as colonies aged and pH values dropped. The pigment was not thought to influence growth, as colonies grown on mannitol containing media produced a very deep pigmentation,

but were above average in dry weight (see Chapter 5.34.2).

It is interesting to note that optimum germination of U. dianthi urediospores and subsequent germ-tube growth occurred in a medium of pH 5.20 (see Chapter 3.32). The urediospore germination process therefore occurs in hydrogen ion concentrations unsuitable for initial saprophytic growth. This would indicate two different pH-optimum metabolisms. If one assumes that the saprophytic and parasitic hyphae of U. dianthi have a similar metabolism, it seems that the change of metabolisms would have to take place between the penetration phase (germination, germ-tube growth, appressorial formation and penetration peg development) and the infection phase (parasitic development within the host). The change therefore would have to be located in the sub-stomatal vesicle, and would perhaps be stimulated by the pH of the plant tissue environment.

CHAPTER 7. SYSTEMIC FUNGICIDE STUDIES ON U. DIANTHI IN VITRO.

7.1 INTRODUCTION.

The particular advantages systemic fungicides have over protectant types lie in their ability to protect new plant growth, offset imperfections in spray application, and in the promise they show in combatting deep seated infections. To act as a systemic fungicide, a compound must be non-phytotoxic, be readily translocated in the host's vascular system and be relatively persistent. When a screening program is carried out, it is necessary to investigate these and other properties. Sometimes the host's metabolism alters the structure of a compound rendering it less toxic or more toxic. A knowledge of the action of the pathogen's metabolism on the compound is also desirable, and also the mode of action of the fungicide. Although intense *in vivo* screening programs are generally needed to investigate the majority of the systemic fungicide properties, *in vitro* tests are still useful, especially in mode of action studies. This in the past has proved difficult with obligate parasites as changes in their metabolism have been almost impossible to distinguish from those of their hosts. *In vitro* experiments also give a quick and easy assessment of the initial activity of a systemic fungicide. If fungicidal activity is high, it is likely that they would act as conventional protectant fungicides in addition to their systemic effects (Byrde, 1969).

It was thought that axenic cultures of *U. dianthi* could be used to determine the relative *in vitro* activities against rusts of new systemic fungicides. To test the plausibility of this, the activities of three different systemic fungicides were compared. These were 1) 'Benlate' (known to have a wide range of activity, controlling many diseases), 2) 'Milstem' (known to

have a limited range of activity, controlling only powdery mildew of cereals), and 3) 'Hoe 6053' (also known to have a limited range, controlling smuts and rusts).

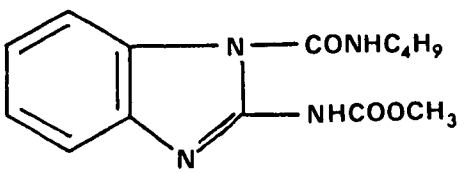
7.2 MATERIALS AND METHODS

7.21 Systemic fungicides.

7.21.1 'Benlate' (Benomyl fungicide).

Manufactured and marketed by Du Pont (U.K.) Ltd., Industrial and Biochemical Department, Du Pont House, 18 Bream's Building, Fetter Lane, London E.C.4.

Active ingredient:- Benomyl (Methyl 1 - (butylcarbamoyl)) 2 - benzimadazolecarbamate. 50%

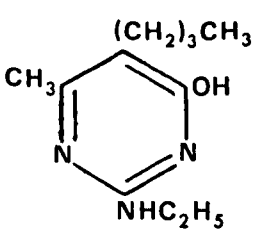


Inert ingredients:- 50%

7.21.2 'Milstem' (Ethirimol Fungicide).

Manufactured and marketed by Imperial Chemcial Industries, Plant Protection Ltd., of Jealott's Hill Research Station, Bracknell, Berkshire.

Active Ingredient:- 5-butyl-2-ethylamino-6-hydroxy-4-methylpyrimidine. 80%

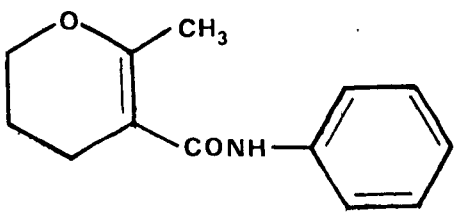


Inert ingredients:- 20%

7.21.3 'Hoe 6053' (Pyracarbalid Fungicide).

Manufactured by Hoechst Chemicals, Hoechst UK Ltd., Hoechst House, Salisbury Road, Hounslow, Middlesex.

Active ingredient:- 2 methyl-5, 6-dihydro-4-Hpyran-3-carboxylic acid anilide. 75%



Inert ingredients:- 25%

7.22 Experimental methods.

Because of the unstable nature of some systemic fungicides, it was decided not to autoclave those used with the growth media. Solutions of fungicides were therefore sterilised by Millipore filtration.

7.22.1 Preparation of media.

The agar medium used was essentially a 'N' medium (see Chapter 5.21.2). A solution containing 36 g Difco Czapek Dox Broth and 5 g Evans' Peptone per litre of distilled water (pH 5.90) was prepared, and 50 ml portions measured by a burette into 250 ml flasks containing 1 g Difco Bacto agar. This was autoclaved for 15 min at 120°C.

Systemic fungicides were weighed very carefully on Oertling R20 balances, and double strength solutions prepared with burette measured volumes of distilled water in 250 ml beakers. After mixing thoroughly to ensure all the fungicide had dissolved, 50 ml of the freshly prepared fungicide solutions were run into

Millipore Sterifil Filtration units and filtered under vacuum through filter discs (0.1 μm diameter pore). Immediately after filtration, which removed the inert material, the solutions were added to the autoclaved media. After swirling, to ensure adequate mixing, three plates were poured per fungicide concentration. The pH of the agars was 6.0.

7.22.2 Subculturing.

Fragments of colonies from 'N' medium stocks were subcultured directly on the agar surfaces. Three subcultures were placed on each plate. All plates were incubated at 18 ^{o}C for one week.

7.22.3 Assessment of fungicidal activity.

Colonies on plates containing different amounts of fungicides were examined microscopically for growth. A subculture was considered to be growing when hyphae were observed actively colonising the medium. The concentrations of fungicide in the media that supported and sustained growth were noted. Usually all nine of the subcultures on each fungicide concentration either established themselves on the media or died.

7.3 RESULTS.

7.31 'Benlate'.

Subcultures grew and formed colonies on plates containing less than 0.17 g of 'Benlate' per litre of the medium. On plates containing more than 0.18 g of 'Benlate' per litre of medium, subcultures failed to establish themselves. The critical level of growth inhibition in the medium was therefore, between 170 and 180 p.p.m. of 'Benlate'. As 50% of 'Benlate' is inert material, the level of benomyl necessary to prevent growth in the medium was between 85-90 p.p.m.

7.31 'Milstem'.

Subcultures established themselves on plates containing

0.80 g of 'Milstem' per litre of medium, but not on plates containing 0.85 g. This growth inhibition level in the medium was therefore between 800 and 850 p.p.m. of 'Milstem', or between 640-680 p.p.m. of ethirimol ('Milstem' 20% inert).

7.33 'Hoe 6053'.

Colonies grew from subcultures on a medium containing 0.001 g of 'Hoe 6053' per litre but not on a medium containing 0.002 g/1. 'Hoe 6053' therefore inhibited growth between 1 and 2 p.p.m. of medium and the active ingredient between 0.75 and 1.5 p.p.m.

7.34 Conclusions.

The systemic fungicide specifically active against rusts and smuts, 'Hoe 6053' was by far the most effective *in vitro*. 'Benlate' the wide range fungicide was moderately effective, while the powdery mildew specific fungicide, 'Milstem' was comparatively ineffective. (Table 7.1).

7.4 DISCUSSION.

The results of the experiment were as expected, the rust specific fungicide inhibiting growth in low concentrations, the wide range fungicide in higher concentrations, while relatively high concentrations of the powdery mildew specific fungicide were needed for inhibition. The relative effects of the fungicide *in vitro* were similar to those that one would expect *in vivo*. It seemed therefore that *U. dianthi* cultures could be used to compare and assess the activities of fungicides against rusts *in vitro*. As mentioned previously however, *in vivo* trials would still be necessary to investigate translocation properties, etc.

The results suggest that 'Hoe 6053' could be used effectively as a protectant as well as a systemic fungicide, as very

TABLE 7.1 A comparison of the activities of three systemic fungicides against U. dianthi in vitro.

Fungicide	% of Inert Material	% of Active Ingredient	Inhibition Level of Fungicide (p.p.m. of 'N' medium)	Active Ingredient	Inhibition level of Active Ingredient (p.p.m. of 'N' medium)
Benlate	50	50	170-180	Methyl 1-(butyl carbomoyl) 2-benzimadazolecarbamate	85-90
Milstem	20	80	800-850	5-butyl-2-ethylamino-6-hydroxy-4-methyl pyrimidine	640-680
Hoe 6053	25	75	1-2	2 methyl-5,6-dihydro 4-Hypran-3-carboxylic acid anilide	0.75-1.5

small quantities were required to inhibit rust growth.

At the moment 'Hoe 6053' is used mainly as a seed dressing, but it is possible that it would be economical to spray solutions of rust- and smut-threatened, and infected crops. Another formulation called 'Hoe 6052' has in fact been prepared for use for foliar application. This is a wettable powder and contains 50% of the active ingredient, pyracarbalid.

Several criticisms can be levelled at this in vitro technique for assessing fungicidal strength. For example, it is possible that in using a solid medium, some of the fungicides were absorbed by the agar. If absorption were uniform with the various fungicides however, the results would still be comparable, and give a good indication as to their relative abilities. In the case of 'Hoe 6053', inhibition was obtained at such low concentrations, that it was thought unlikely that much absorption could have occurred, either by the agar, or by the carbohydrate or nitrogen sources in the media. Stott (1971) found using 'Fungex' and 'Captan' that the addition of carbon sources to media had no significant effect on the minimum inhibitory doses of the fungicides.

The removal of some of the active ingredient during Millipore sterilisation was also possible. Again the results with 'Hoe 6053' indicated that it is unlikely this occurred in this case. This is probably because all active ingredients in systemic fungicides are water soluble for translocation reasons.

Although no mode of action studies were undertaken with the fungicides on axenic cultures of U. dianthi, work of this kind is possible. In the future research into the chemical basis of fungicidal action on rust metabolism may be realised through in vitro culturing.

CHAPTER 8. ELECTRON MICROSCOPE STUDIES ON SAPROPHYTIC U. DIANTHI HYPHAE.

8.1 INTRODUCTION.

In the past little work seems to have been published on the ultrastructure of obligate parasites, probably because of the difficulties workers must have experienced with resin infiltration and sectioning due to the presence of the host plant tissues. The successful axenic culture of U. dianthi provided an opportunity for the examination of rust hyphae free from host plant material.

A problem hitherto unresolved was that of the septa and septal pores in the Uredinales. This was selected as the primary objective of the electron microscopic study.

Moore & McAlear (1962) proposed that the septal pore could be used as an ultrastructural morphological feature to distinguish the Basidiomycetes from the Ascomycetes. The Basidiomycetes investigated possessed elaborate dolipore-parenthosome septal pores (Fig. 8.1) whereas the Ascomycetes were shown to have simple (central clear channel) pores (Fig. 8.2). The dolipore-parenthosome pore was subsequently reported in other Basidiomycetes (Bracker & Butler, 1963; Wells, 1964; Berliner & Duff, 1965; Manocha, 1965; Wells, 1965; Wilsenach & Kessel, 1965; Hyde & Walkinshaw, 1966; Niederpruem & Wessels, 1969).

This type of pore was however not found in Puccinia podophylli (Moore, 1963), P. graminis f. sp. tritici (Ehrlich & Ehrlich, 1963) or M. lini (Manocha & Shaw, 1967). These reports suggested that the complex 'dolipore-parenthosome' pore was not characteristic of all Basidiomycetes.

Ehrlich, Ehrlich & Schafer (1968) working with P. graminis and P. recondita confirmed this, and found that simple pores in young hyphae of these rusts are characterised by the presence

Fig. 8.1. A typical dolipore-parenthosome septal pore arrangement (after Moore & McAlear, 1962).

Fig. 8.2. A typical simple septal pore arrangement (after Moore & McAlear, 1962).

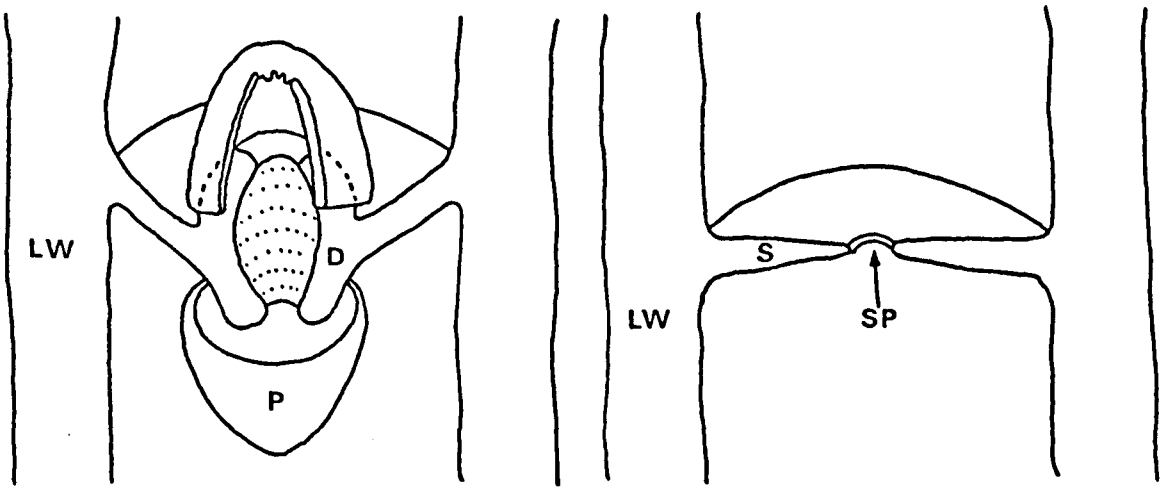
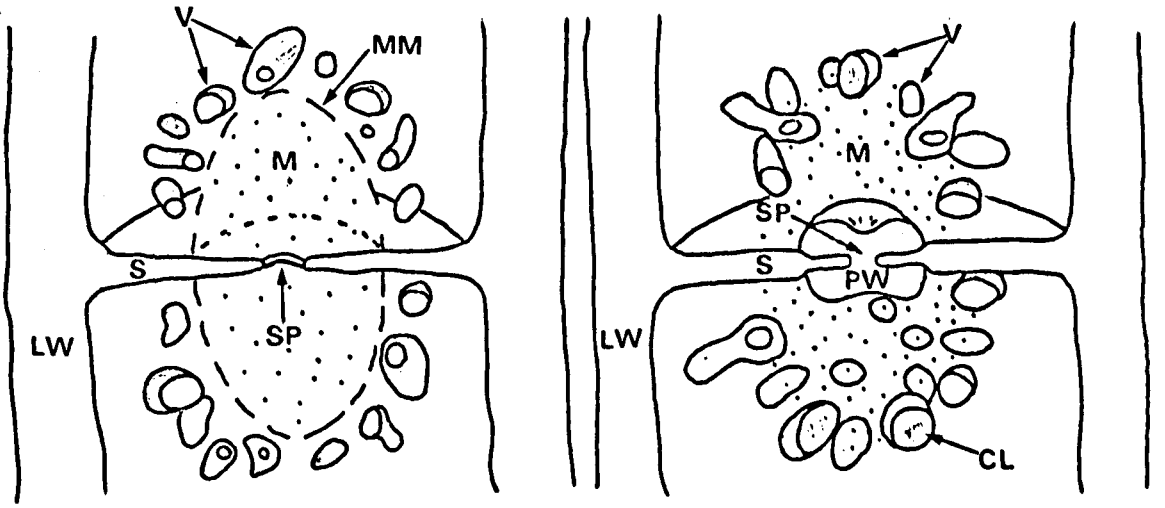


Fig. 8.3. Cytoplasmic matrix septal pore arrangement (Ehrlich et al., 1968).

Fig. 8.4. Pulley wheel plug septal pore arrangement (after Littlefield & Bracker, 1971).



Key. CL, crystalline lattice observed in some vesicles; D, dolipore septum; LW, longitudinal wall; M, cytoplasmic matrix around septal pore, usually free of organelles; MM, incomplete matrix membrane; P, parenthosome; PW, pulley wheel septal pore plug; S, septum; SP, septal pore; V, peripheral vesicles.

of a cytoplasmic matrix (Fig. 8.3). This matrix apparently occluded the pore preventing the migration of organelles from cell to cell. It was thought that the matrix may have been incompletely bounded by a membrane. Large vesicles were also observed at its periphery. It was suggested that the matrix acted as a plug in young cells and disappeared, together with its associated vesicles, in older cells, leaving a septum indistinguishable from the typical Ascomycete-type simple pore.

Large openings were seen in some septa which allowed nuclei and other bodies to migrate from cell to cell. This was thought to be the result of a breakdown of a complete septum. Pseudosepta produced by lateral wall infoldings and limited septum formation were also observed.

Recently Littlefield & Bracker (1971) described septa in the filamentous hyphae of M. lini partially to completely blocked by electron dense plugs with the three dimensional configuration of pulley wheels (Fig. 8.4). In cells judged to be mature, the plug either had the appearance of a narrow dark band surrounding the pore area or consisted of accumulations of electron dense material on one or both sides of the pore. The plug was often surrounded by a distinct region of cytoplasm devoid of organelles and resembled the matrix described by Ehrlich et al. (1968). In senescent cells, the plug appeared dispersed and only partially blocked the septa. Partial or pseudo-septa were also observed in filamentous hyphae, and complete septa without pores, were found in pseudoparenchyma of uredial primordia.

The septal pore structures in the Basidiomycetes have been proposed to have possible phylogenetic significance, (Moore, 1965; Wilsenach & Kessel, 1965). The dolipore-parenthosome type was said to be more advanced than the matrix types found in the Uredinales which were essentially modified

Ascomycete simple pores. The septum breakdown mechanisms of P. graminis and P. recondita however were known to occur in phylogentically more advanced Basidiomycetes. This mechanism has not been recorded in Ascomycetes (Ehrlich et al., 1968). This places the Uredinales phylogenetically between the Ascomycetes and Basidiomycetes.

The septal pores of U. dianthi were investigated to determine if the matrix and pulley wheel plug existed in this rust. It was thought that improved techniques on saprophytic hyphae would clarify the problem of this phylogenetically intermediate pore mechanism.

8.2 MATERIALS AND METHODS.

8.21 The saprophytic hyphae used in the investigation.

The U. dianthi colony used was growing directly on the surface of a 'N' medium agar plate. It had been subcultured 6 weeks previously from U. dianthi 'N' medium stocks (see Chapter 5.21.2), and incubated in the dark at 18 ^{o}C. Samples of saprophytic hyphae were taken from three different areas.

Colonizing surface hyphae.

The marginal hyphae in contact with the agar surface on the periphery of the colony were broken into small rectangular pieces (approximately 3 x 2 mm) using sharp scalpel blades.

Aerial hyphae.

Mycelial segments (approximately 2 x 2 mm) were cut from the crown of the colony. For these samples to be taken, the colony was lifted from the agar surface and fragmented in a clean Petri dish.

Submerged hyphae.

After the removal of the surface portion of the colony, small blocks (approximately 3 x 3 mm) containing strands of

submerged hyphae were cut out of the agar.

The portions of mycelium were transferred directly into fixative.

8.22 Fixatives and embedding resin

Formaldehyde/gluteralderhyde fixative

1% (v.v.) Formaldehyde (made up from paraformaldehyde powder according to the method of Pease, 1964).

3% (v.v.) Gluteralderhyde in 0.2 M sodium cacodylate buffer (pH 6.5).

Osmium fixative

1% (v.v.) Osmium tetroxide (OsO_4) in 0.1 M phosphate buffer (pH 6.1).

Embedding resin

Spurr's embedding resin was used because of its superior inflitration properties (Spurr, 1969). The formulation was:-

ERL-4206 (vinyl cyclohexene dioxide) 35 g.

DER-736 (diglycidyl ether of polypropylene glycol) 15 g.

NSA (nonenyl succinic anhydride) 65 g.

S-1 (dimethylamino ethanol or DMAE) 1 g.

The medium was prepared by dispensing the components into a stoppered bottle by means of plastic syringes. The bottle was continually weighed on a balance to ensure that correct amounts of the components were added. The relatively low viscosity of the medium permitted rapid mixing by shaking and swirling.

8.23 Fixation, dehydration and infiltration Procedure.

Fixation, dehydration and infiltration were carried out in 15 ml glass phials with polythene caps.

The mycelial pieces were initially fixed in formaldehyde/gluteralderhyde fixative for 1½ h. After a ½ h buffer wash (0.2 M sodium cacodylate, pH 6.5) the segments were transferred to osmium fixative for ½ h. After a distilled water rinse, the

fragments were stained for 5 min in a 2% aqueous uranyl acetate solution. They were then transferred to a 30% acetone solution (5 min) then to 60% (5 min), 90% (5 min), and 100% (2 x 5 min) acetone solutions. Agitation during the fixation and dehydration process was provided by a Luckham's swirling shaker. The samples were then placed in an acetone resin 3:1 mixture for 30 min. followed by 30 min in a 1:1 mixture and 30 min in a 1:3 mixture. After two changes of 100% resin ($\frac{1}{2}$ h in each), the pieces were left in fresh 100% resin overnight (16 h). A Matburn blood cell suspension miser was used to agitate the phials during infiltration.

8.24 Embedding, sectioning and staining procedure.

The ends and lids of TAAB embedding capsules were cut off using a sharp scalpel. The caps were then partially filled with resin and the mycelial fragments arranged in the caps so that the majority of the hyphae were lying parallel to the base (the hyphae when sectioned would then be cut in a longitudinal plane rather than a transverse one and would enable better observations to be made on septal pores). The main body of the capsule was then fitted into the cap, and filled almost to the top with resin. The capsules were left in this inverted position in a 60°C oven. After two days, the hard resin blocks were cut from the capsules and sectioned on a Reichert OMU2 microtome. The sections were cut with glass knives made on a L.K.B. Knifemaster, Type 7801A. Sections that appeared silver in colour (approximately 600 nm thick) were placed on Athene 200 mesh grids.

The grids with sections were stained by floating on single drops of 5% saturated aqueous uranyl acetate solution for between 5 and 10 min. This was followed, after a distilled water rinse, by 5 min on drops of lead citrate solution (Reynolds, 1963). The sections were then washed in distilled water and

placed on filter papers in Petri dishes until used.

8.25 Electron microscope photography.

The electron microscope used was a Phillips E.M.200 with a 50 μm objective aperature which was operated at 60 kV.

Extensive searches were necessary to locate sections through septal pore areas. Photographs were taken at various magnifications; the electron optical exposure used was 3 mV s (1.5 mV for 2 s) and the film was Recordak microfile 35 mm film type 5669. The film was developed using Kodak D19 developer for 4 min at 20°C and prints made on Kodak Bromide 'K3' paper.

8.3 RESULTS.

Light microscope studies showed hyphal cells of various physiological 'ages' often adjacent to one another. Most cells contained very dark staining lipid bodies. Occasionally cells with darker staining cytoplasm were observed.

Electron microscopic searches for submerged hyphae was difficult and time consuming because of the sparse number in any one section. For this reason, observations were concentrated on sections where hyphae were fairly densely grouped. The most rewarding sections were ones cut from blocks of aerial hyphae. All photographs of pore areas were taken on these sections.

Initial observations confirmed that the cells of the saprophytic hyphae possessed two lobed nuclei (Pl. 8.1).

8.31 The Septal pore.

When observed, the septal pores were always located in the central areas of the septum (Pl. 8.1). The pore was usually formed by a tapering of the septum to the pore margin (Pls. 8.2, 8.3). The septum was often slightly wider than average before the tapering occurred (Pl. 8.4). On thicker walls this tapering could be much more pronounced on one side of the septum,

giving the impression of an indentation in the septal wall (Pl. 8.5). This seemed to be caused by the unequal addition of septum material from the hyphal cells; one side of the wall being built up more than the other. Occasionally, the septum rounded off at the pore area, the margins being less electron dense than the rest of the septum (Pl.8.6).

The septal pores varied in diameter from 500 nm to 1000 nm, and septal wall thickness from 600 nm, to 3,300 nm.

8.32 The Cytoplasmic matrix

In one observed septal pore area, the pore on one side of the septum was surrounded by a cytoplasmic matrix, distinctly different from the surrounding cell cytoplasm (Pl.8.3). The matrix did not appear to be membrane bound, and no inclusions were seen in it. Electron dense single membrane bound vesicles were however located on its periphery (Pl. 8.3). This resembled the pore arrangement observed by Ehrlich et al., (1968) in P. graminis and P. recondita, and Littlefield and Bracker (1971) in M. lini.

8.33 Electron dense bands.

Electron dense bands were observed on the septal walls in many sections and appeared to be associated with the inner layer of the plasmalemma. In some sections the band was well defined and was observed to separate from the plasmalemma (to a distance of approximately 300 nm) in the septal pore area (approximately 1000 nm from the pore), and was continuous over the pore, apparently forming a barrier (Pls. 8.3, 8.7). However, because of its centrally indented shape it was believed that the band formed a narrow inter-cellular corridor through the pore (interpretation of non-median sections).

The shape of the indented dark bands resembled the bands found by Littlefield & Bracker (1971) around the septal pores

of M. lini. As in M. lini, they were not regarded as double or unit membranes as they lacked the typical dark-light-dark staining pattern.

8.34 Peripheral vesicles.

In many sections, a distinct matrix could not be definitely discerned around the pore areas, but electron dense single membrance bound spheroid and elongated vesicles were present in a peripheral position (Pl. 8.8). In some sections, the raised electron dense band associated with the pore was present (Pl. 8.7). Some vesicles were observed to contain structures of regular linear patterning, indicating a crystalline interior (Pls. 8.3, 8.5, 8.9). Often sections near pore areas revealed masses of vesicles (Pl. 8.10). From their situation it appeared that they were distributed in roughly a dome-shaped layer over the pore area.

8.35 Vesicles and the septal pore.

In one section, two dark peripheral-type vesicles appeared very close to the septal pore. In the adjacent hyphal cell, also very near the pore, an apparently empty vesicle with a ruptured wall was observed. An electron dense substance, much darker than the surrounding cytoplasm, occupied the area between the ruptured wall and the actual septal pore (Pls. 8.4, 8.11). In this section, the cytoplasmic matrix could just be distinguished on one side of the pore, as it appeared slightly darker than the surrounding cytoplasm, and was devoid of the numerous dark glycogen granules and endoplasmic reticulum (Pl. 8.4).

Another section revealed an electron dense substance apparently passing through a septal pore (Pl. 8.12). This was closely associated on one side of the septum, with electron dense vesicles (Pl. 8.9).

In a section containing physiologically 'older' cells, an electron dense vesicle was seen in close contact with a

septal pore. In the adjacent cell, an empty ruptured vesicle, possibly a continuation of the full vesicle, was observed (Pl. 8.5).

8.36 Pore plugs.

Many septal pores appeared filled with dark electron dense material (Pls. 8.2, 8.6, 8.13). Generally, though not always, one or both of the cells were physiologically mature and contained large vacuoles and numerous lipid bodies. The cytoplasmic pore matrix was usually absent in these plugged cells, though a few faint vesicles could be still discerned (Pls. 8.2, 8.6, 8.13).

In some sections the plugs appeared centrally indented (Pls. 8.2, 8.6), and of similar shape to the electron dense bands observed across the pore in the peripheral vesicle arrangement (Pls. 8.3, 8.7). This indentation was again indicative that the plugs may not completely occlude the pore (interpretation of a non-median section).

More evidence for this theory came from two sections in which similar large double membraned cell vesicles were observed. In one section the vesicles appear on either side of the septum and seem separate entities (Pl. 8.14). In the other section, which was next but one to the first section (i.e. approximately 600 nm from it), the vesicles converge on one another at an apparently plugged pore (Pl. 8.15). Although it could be argued that the formation of the plug severed the large vesicle as it was passing through, or even that the two vesicles were completely independent of one another, it seemed more likely that a possible connection between the two lay just out of the place of the section. If this interpretation is correct, electron dense plugs may have very small corridors through them. It also demonstrates the flexibility of large cell inclusions.

The break in the septal wall on these sections was not natural because of the rugged edges of the septum, and complete electron transparency of the hole. It was considered to have been induced by the handling of the hyphae during fixation etc.

On no occasion were pseudosepta or natural breakdowns in the septal wall observed.

Large plugs were seen in pores between some dying cells with disorganised contents (Pl. 8.16 , 8.17). In other old cells they appeared absent (Pl. 8.18).

8.4 DISCUSSION.

The absence of the typical Basidiomycete 'dolipore-parenthosome' septal complex in the hyphae of U. dianthi, was in agreement with the findings of other rust ultrastructure research workers (Ehrlich & Ehrlich, 1963; Moore, 1963; Manocha and Shaw 1967; Ehrlich et al., 1968 Littlefield & Bracker, 1971).

Because no cell organelles were observed inside the cytoplasmic matrix surrounding the pore areas of P. graminis and P. recondita, Ehrlich et al. (1968) suggested that the matrix served as a plug which prevented the migration of nuclei and other bodies. They also proposed from their observations that as the hyphae aged and lost their contents, the matrix plug and vesicles disappeared, and the septal pore became similar in form to the Ascomycete-type simple pore.

From observations on U. dianthi pore areas, the Ehrlich et al. (1968) theory that the cytoplasmic matrix served as a cell organelle plug (maintaining cell diploidy), seems plausible in the early stages of a rust cell's life. At a certain stage in the life of two adjacent cells however, an imbalance is created which it is believed causes the matrix to disappear

and an electron dense plug to form in the septal pore. This seems to be associated with a differential ageing of the two cells, perhaps creating a nutrient sink or an osmotic difference in one of them. When the imbalance becomes critical and the matrix disappears, observations suggest that some of the peripheral vesicles migrate towards the septal pore and block it.

It has not been possible to deduce just how the mechanism operates, though it seems likely that as the matrix breaks down (usually on one side of the septum before the other, Pl. 8.3), the vesicles move on the inter-cellular flow towards the pore.

The repeating pattern, indicating a crystalline interior that was observed in some vesicles, together with the fact that they do act as pore plugs suggests that they are analagous to the inclusions (Woronin bodies) found in Ascomycetes (Buller, 1933; Dickson, 1963; Schrantz,1964; Denison & Carroll, 1966; Lowry & Sussman, 1966; Bracker, 1968) and Fungi Imperfecti (Reichle & Alexander, 1968).

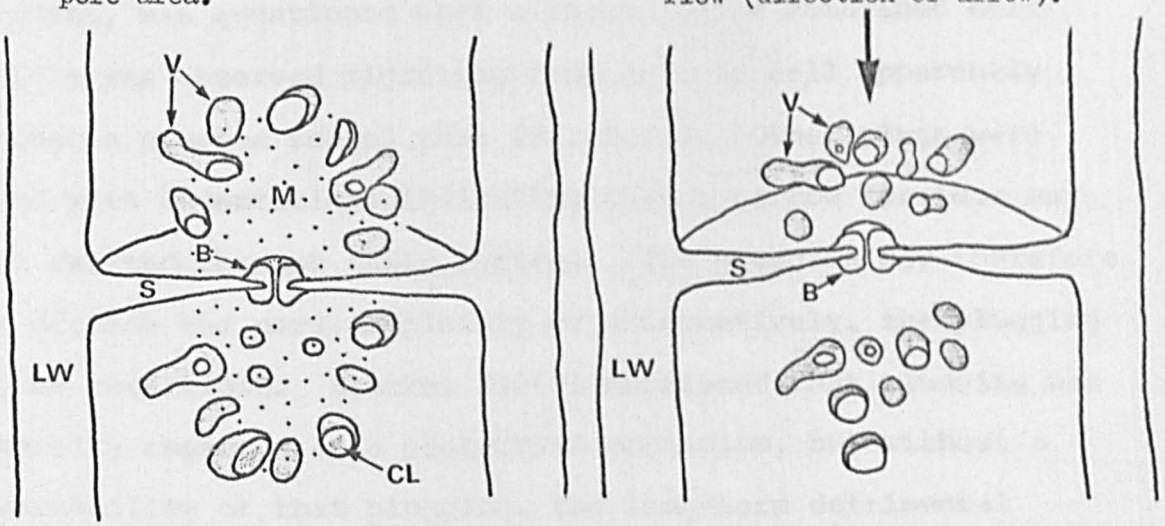
The crystalline material in the vesicle may act to rupture the vesicle membrane as it squeezes through the narrow septal pore aperture on the intercellular flow (Pls. 8.4, 8.5, 8.9, 8.11, 8.12). The interior thus released may coalesce and concentrate in the pore thereby partially or completely plugging it.

This possible plugging process is illustrated in Fig. 8.5.

All observed plugs lacked a visible membrance and were more electron dense than most of the vesicles. This was in contrast a little to the membrane bound plugs of the same electron density as the Woronin bodies found by other workers (Dickson, 1963; Schranz, 1964; Reichele & Alexander, 1965). A change in density due to a concentrating action of the cell

a) Cytoplasmic matrix with peripheral vesicles. Electron dense band conspicuous in pore area.

b) Breakdown of matrix and movement of vesicles towards the septal pore on inter-cellular flow (direction of arrow).



c) Vesicle rupturing and discharging contents as it passes through the septal pore.

d) Formation of an electron dense septal pore plug. Remaining vesicles degenerate.

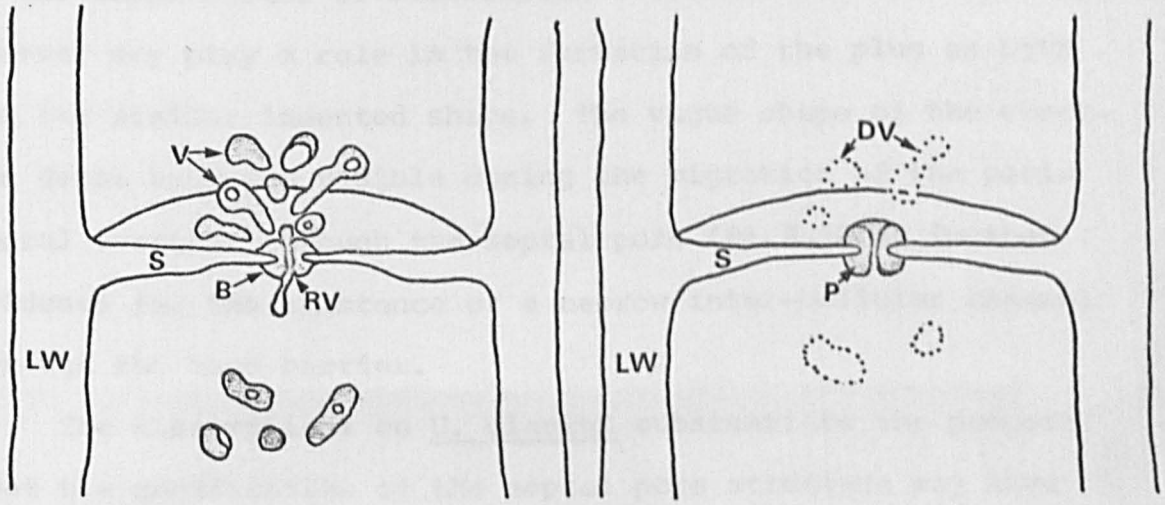


Fig. 8.5a-d. Proposed U. dianthi septal pore plugging mechanism.

Key. B, electron dense band with a narrow channel through the septal pore; CL, crystalline lattice observed in some vesicles; DV, degenerating vesicles; LW, longitudinal wall; M, cytoplasmic matrix; P, electron dense plug with a narrow inter-cellular channel; RV, rupturing vesicle; S, septum; V, peripheral vesicles.

cytoplasm after membrane rupture could explain this.

The efficiency of the plugs in preventing inter-cellular movement, was questioned when a large double membraned cell vesicle was observed migrating from cell to cell apparently through a plugged septal pore (Pl. 8.15). Other plugs were found with indentations indicating that a narrow corridor may have existed through their centres. The plugging may therefore not occlude the pore completely or alternatively, the plugging may be reversible. Bracker (1967) mentioned that plugging was generally regarded as a protective mechanism, but without a reversibility of that plugging, the long-term detrimental effects could outweigh the protective aspects.

The theory presented here is based on electronmicrographs taken of the septal pore regions of U. dianthi filamentous hyphae. No evidence was found that the indented electron dense band increased to form the final electron dense plug as suggested by the observations of Littlefield & Bracker (1971). The band however may play a role in the formation of the plug as both had the similar indented shape. The vague shape of the electron dense band was visible during the migration of the peripheral vesicles through the septal pore (Pl.8.12.), further evidence for the existence of a narrow inter-cellular channel through the band barrier.

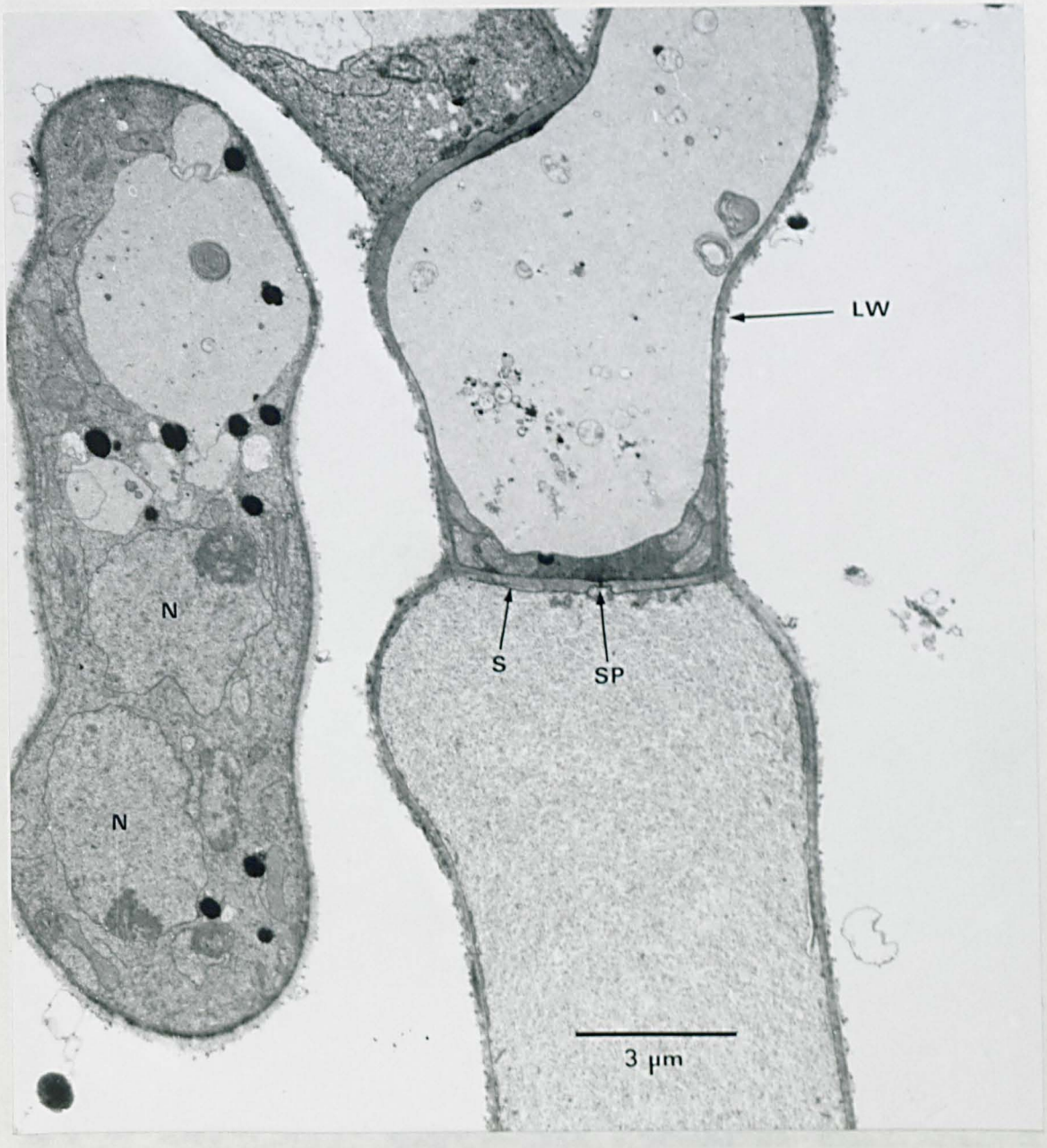
The observations on U. dianthi substantiate the proposal that the modification of the septal pore structure may have phylogenetic significance. The absence of the typical dolipore-parenthosome septal arrangement indicates that the rusts are phylogenetically less advanced Basidiomycetes. Several features, namely the essentially simple pore structure, the presence of vesicles analagous to Woronin bodies, and the electron dense pore plugs are of the Ascomycete-type.

The septum breakdown mechanism of P. graminis and P. recondita (Ehrlich et al., 1968) was not observed in U. dianthi. Similar mechanisms have been reported in more phylogenetically advanced Basidiomycetes (Lehfeldt, 1923; Jersild, Mishkin & Niederpreum, 1967) but are not known to occur in Ascomycetes. This suggests that U. dianthi is phylogenetically less advanced than P. graminis and R. recondita.

All observations were carried out on saprophytic rust hyphae, and it is possible that differences in ultrastructure may exist between U. dianthi cells in vitro and those in vivo.

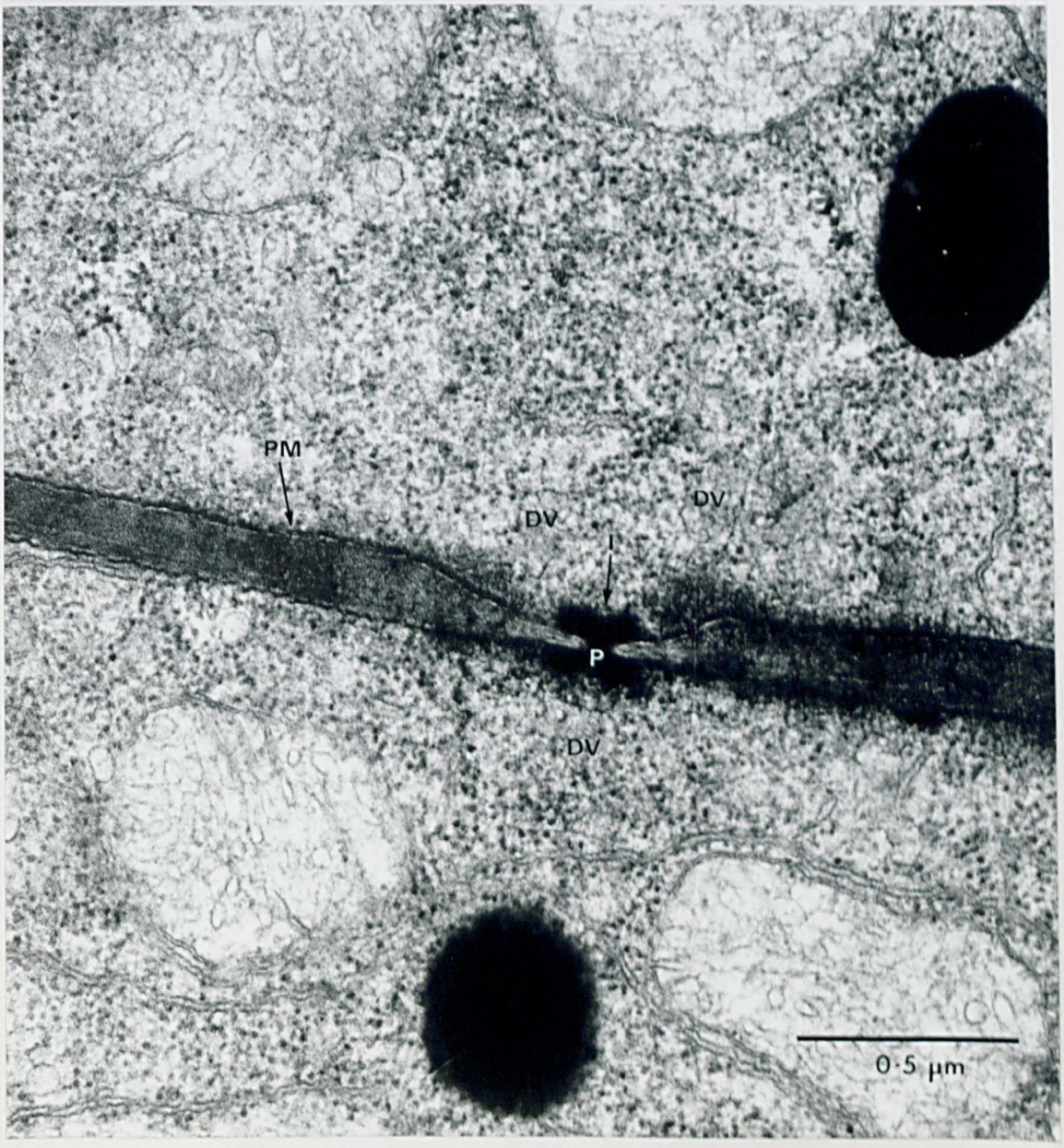
8.5 ELECTRONMICROGRAPHS

Plates 8.1-18. Ultrathin sections through saprophytic *U. dianthi* hyphal cells.



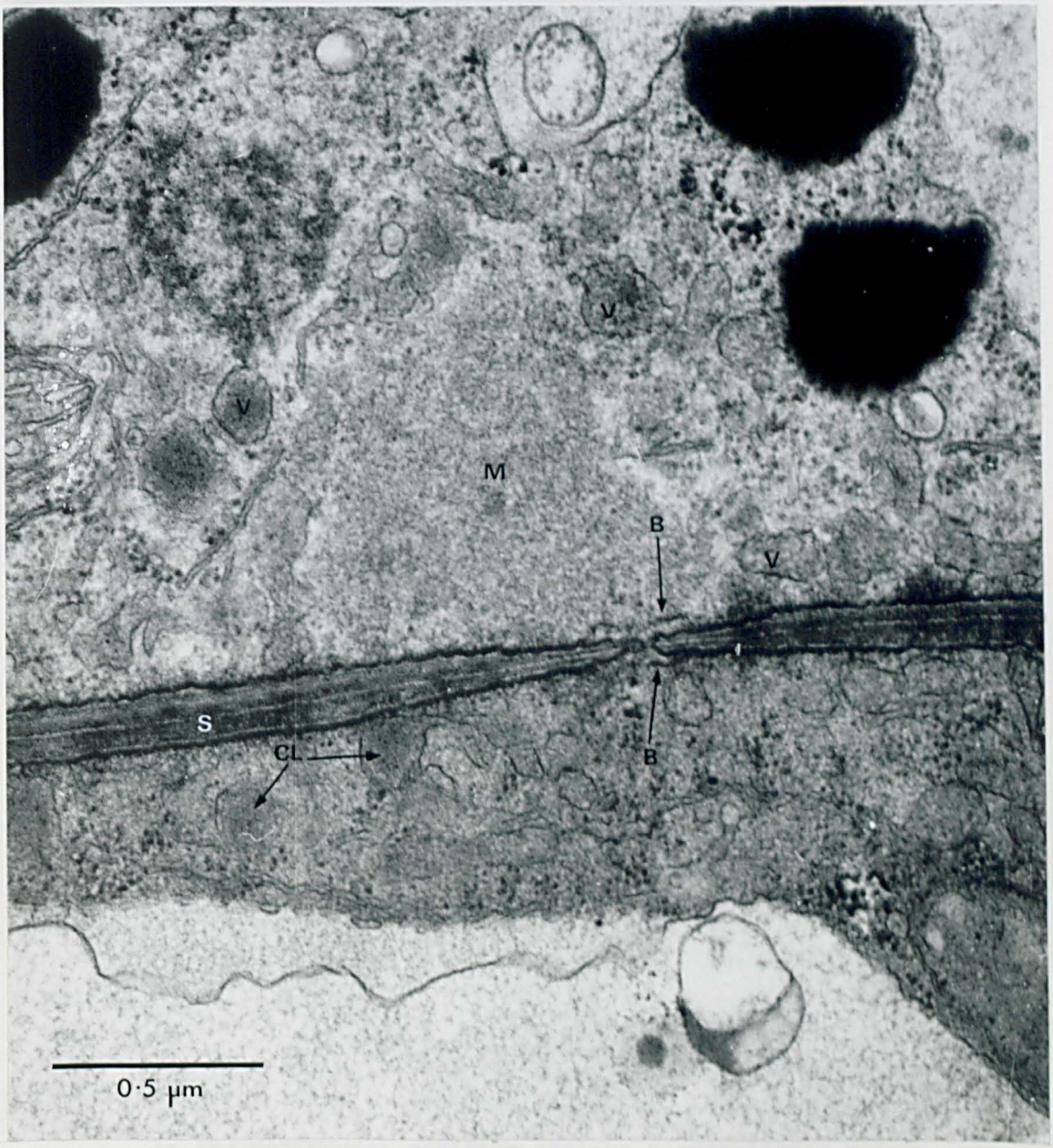
Pl. 8.1 A hyphal cell containing two nuclei (left). Two hyphal cells separated by a septum, showing the position of the septal pore (right). X 7939.

Key. LW, longitudinal wall; N, nucleus; S, septum; SP, septal pore.



Pl. 8.2 A septum tapering to the pore margin. The septal pore is occupied by an indented electron dense pore plug. Degenerating vesicles lie around the pore. X 63804.

Key. DV, degenerating vesicles; I, plug indentation; P, plug; PM, plasmalemma.

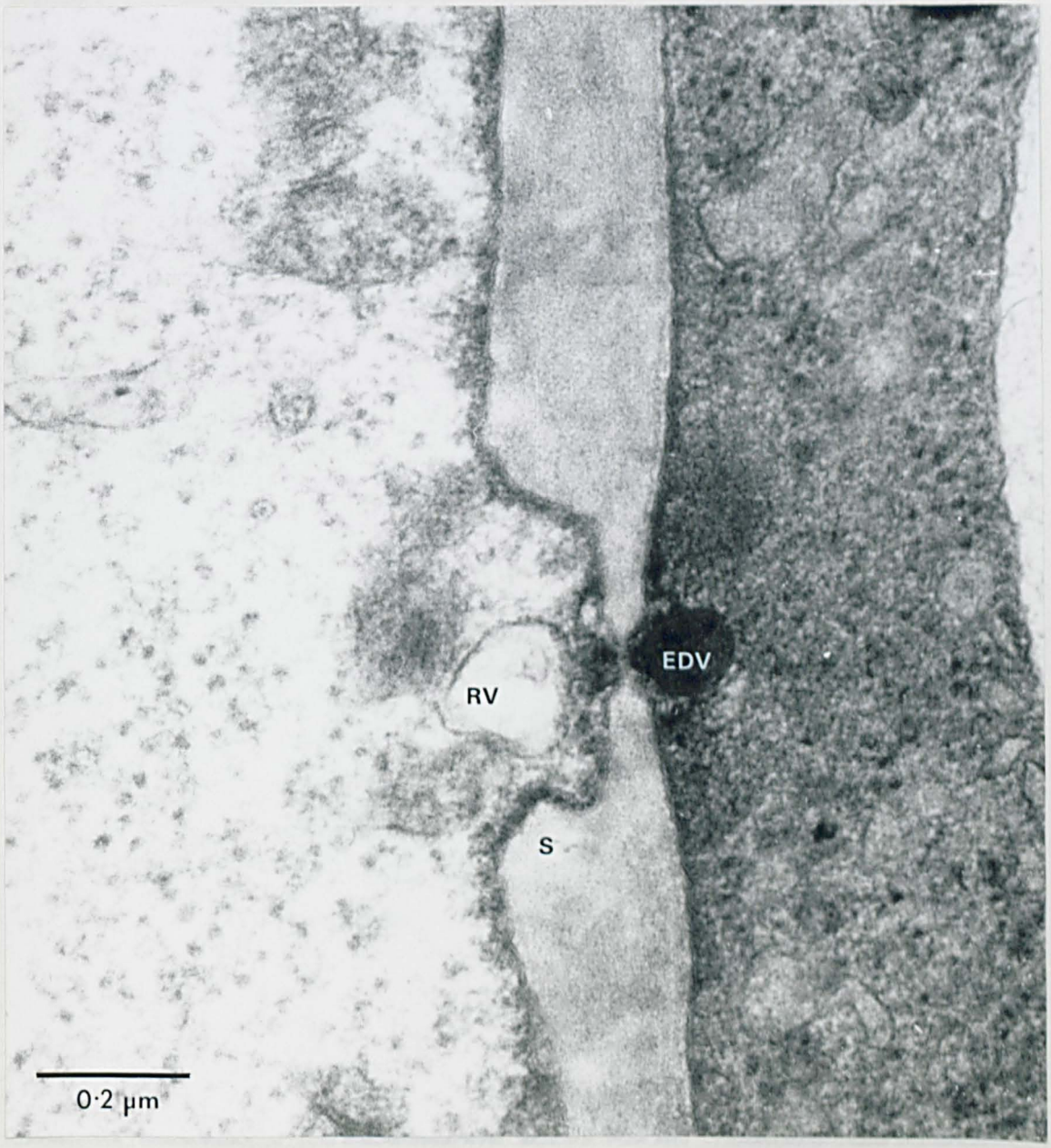


Pl. 8.3 A septum tapering to the pore margin. A cytoplasmic matrix with associated peripheral vesicles is present on one side of the septum. The matrix is not discernible on the other side of the septum, though the vesicles are prominent and nearer to the septal pore. Some contain a repeating pattern indicative of a crystalline interior. Electron dense bands with slight indentations are present across both sides of the septal pore. X 63804.

Key B, electron dense bands; CL, crystalline lattice; M, cytoplasmic matrix; S, septum; V, vesicles.

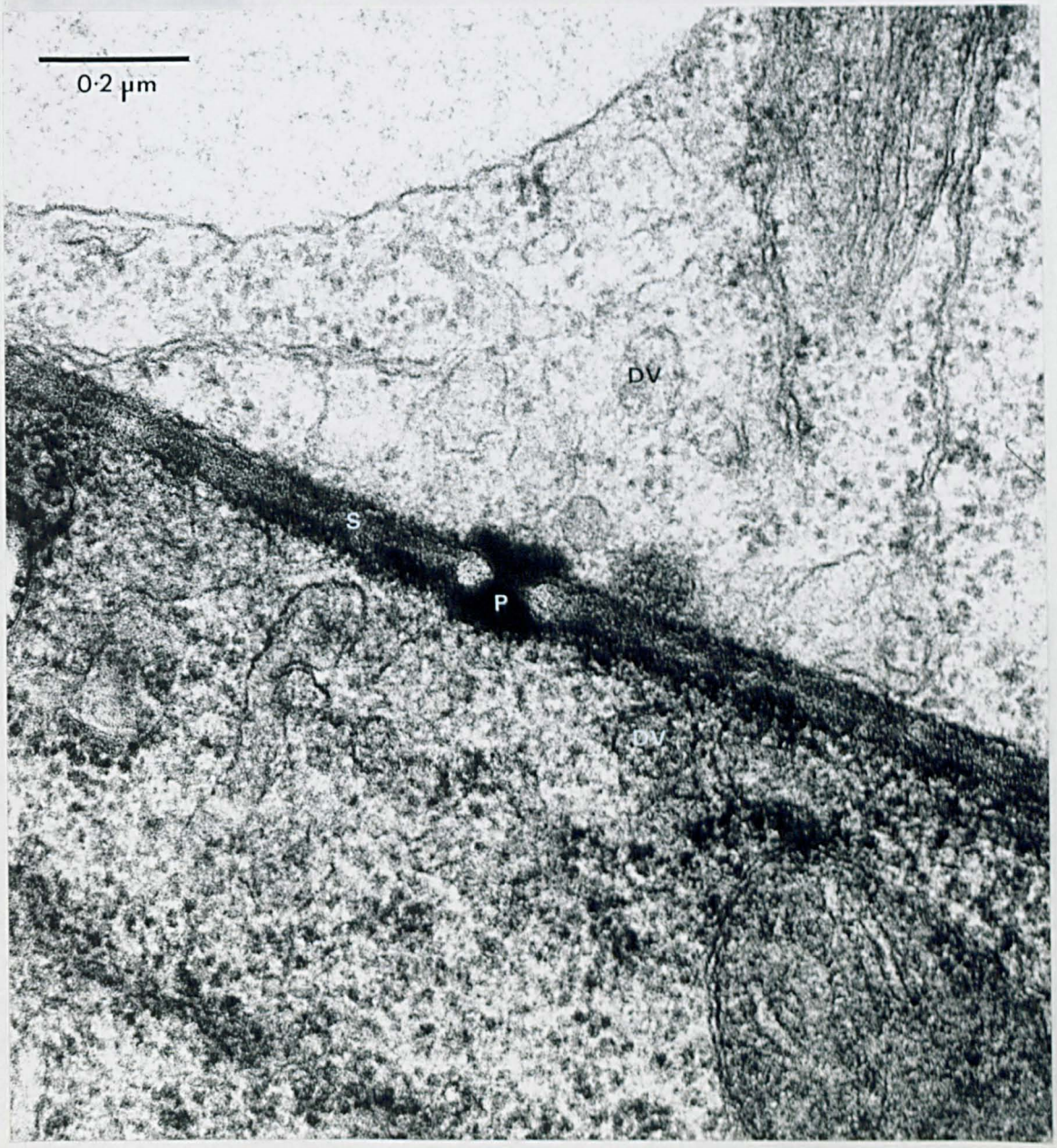
Pl. 8.4 A septum tapering to the pore margin. On one side vesicles in the peripheral position enclose a cytoplasmic matrix (contains no glycogen granules). Patterning, indicative of a crystalline interior, is present in some of these vesicles. Two electron dense vesicles lie close to the septal pore on the other side of the septum. An apparently empty, ruptured vesicle lies very close to the pore on the opposite side of the septum. An electron dense substance lies in the septal pore. X 63804.

Key. CL, crystalline lattice; EDS, electron dense substance; GG, glycogen granules; M, cytoplasmic matrix; RV, ruptured vesicle; S, septum; SP, septal pore; V, vesicles.



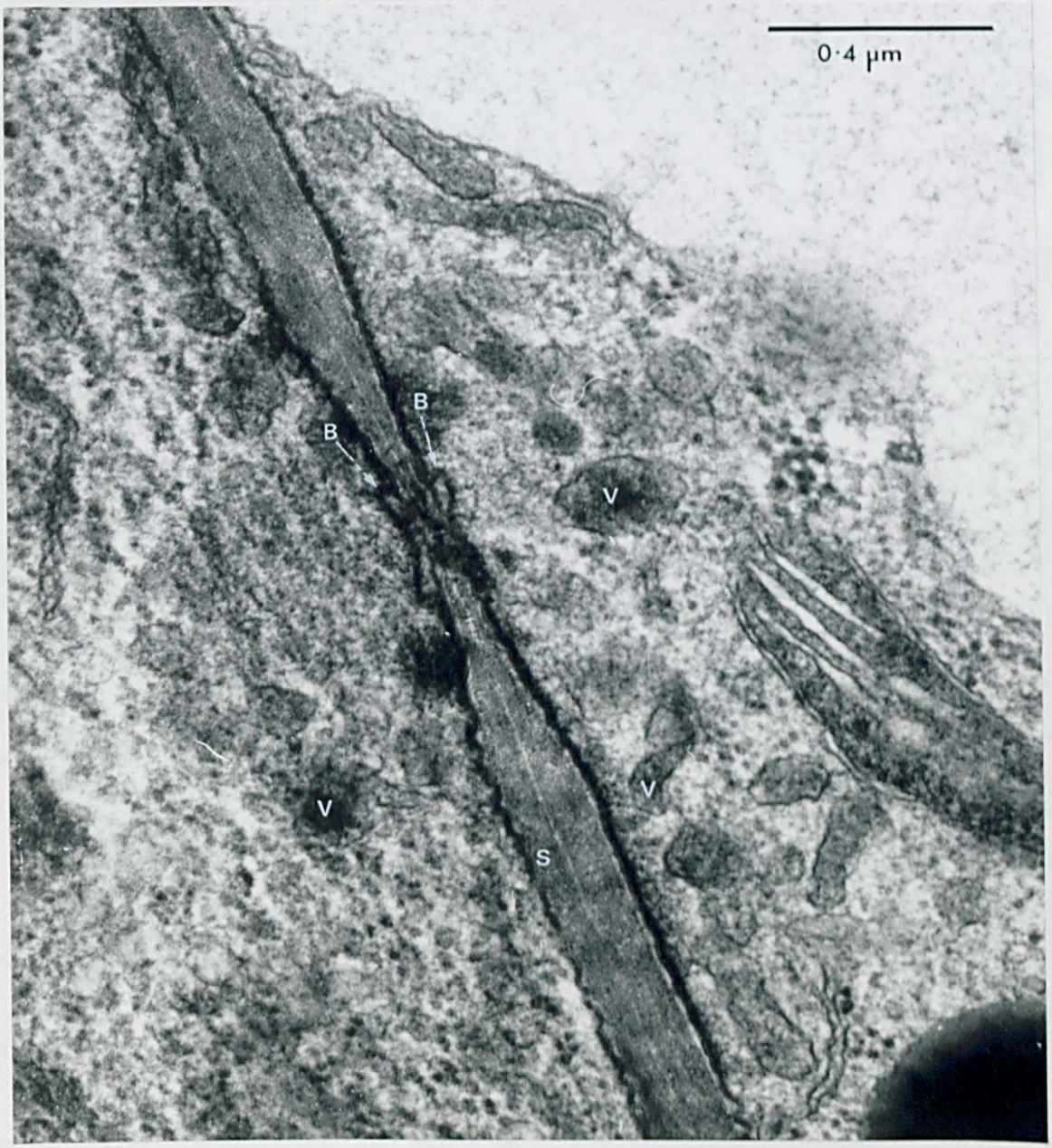
Pl. 8.5 A septum indented at the pore margin. An electron dense vesicle is passing through the septal pore. An empty vesicle with a broken membrane lies on the other side of the pore (possibly a continuation of the electron dense vesicle). X 110617.

Key. EDV, electron dense vesicle; RV, ruptured vesicle; S, septum.



Pl. 8.6 A septum with a rounded, less electron dense margin at the septal pore. An indented electron dense pore plug occupies the septal pore. Degenerating vesicles lie near the pore area. X 110617.

Key. DV, degenerating vesicles; P, pore plug; S, septum.



Pl. 8.7 A raised, indented electron dense band is present over both sides of the septal pore. Vesicles lie around the pore area. X 83750.

Key. B, electron dense band; S, septum; V, vesicles.

Pl. 8.8 Peripheral vesicles surround the septal pore area. The cytoplasmic matrix is not discernible. X 37158.
Key. S, septum; V, peripheral vesicles.

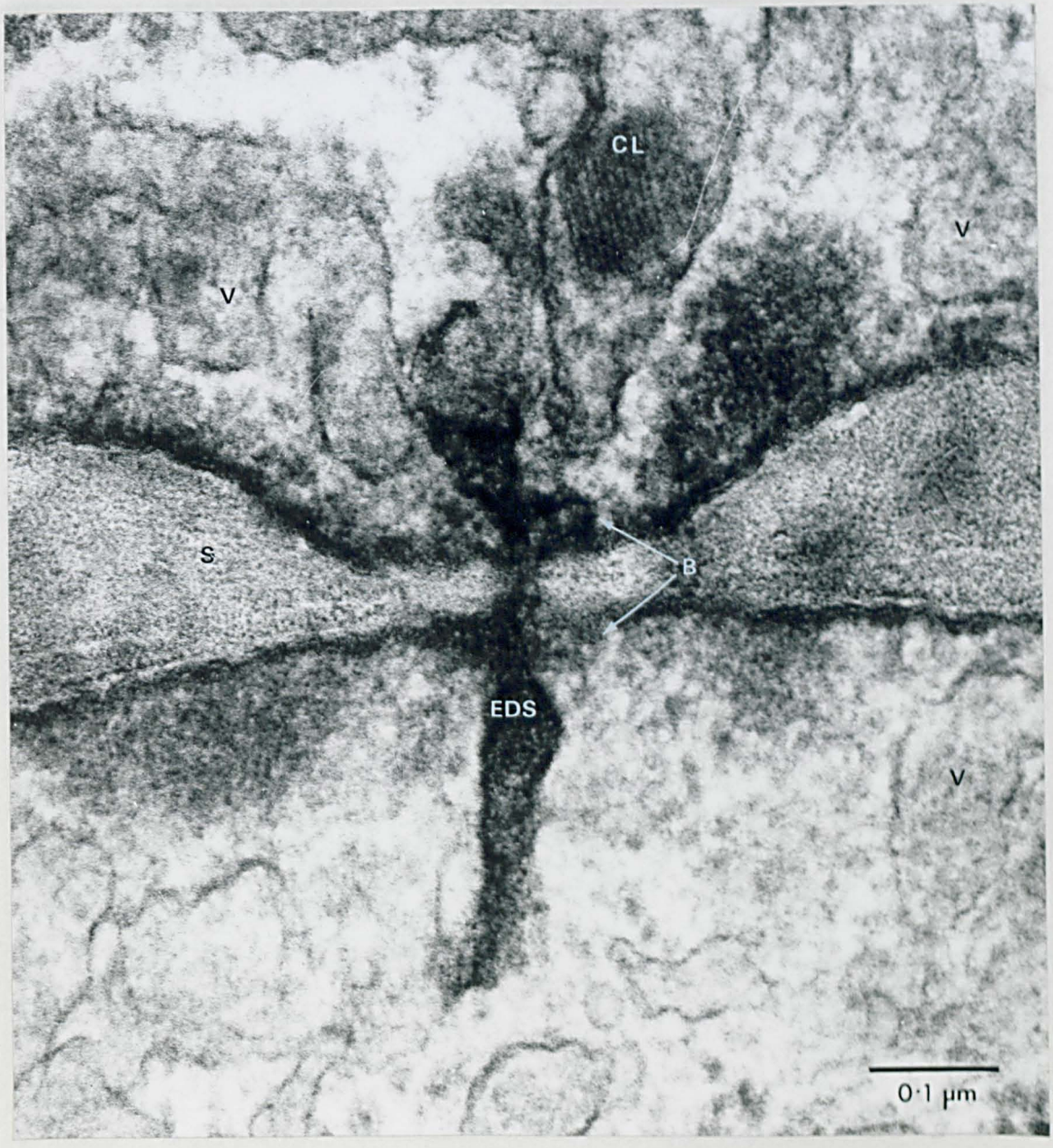
Pl. 8.9 Vesicles, some with a crystalline lattice interior, very near to the septal pore. An electron dense substance (believed to be a vesicle) is passing through the pore. Electron dense bands, which are just visible, do not seem to act as a barrier to its passage. X 63804.

Key. B, electron dense bands; CL, crystalline lattice; EDS, electron dense substance (vesicle); S, septum; V, vesicles.

Pl. 8.10 Vesicles asscociated with the septal pore area. The pore area is indicated by a narrowing of the septum. X 22337. Key. LW, longitudinal wall; S, septum; V, vesicles.

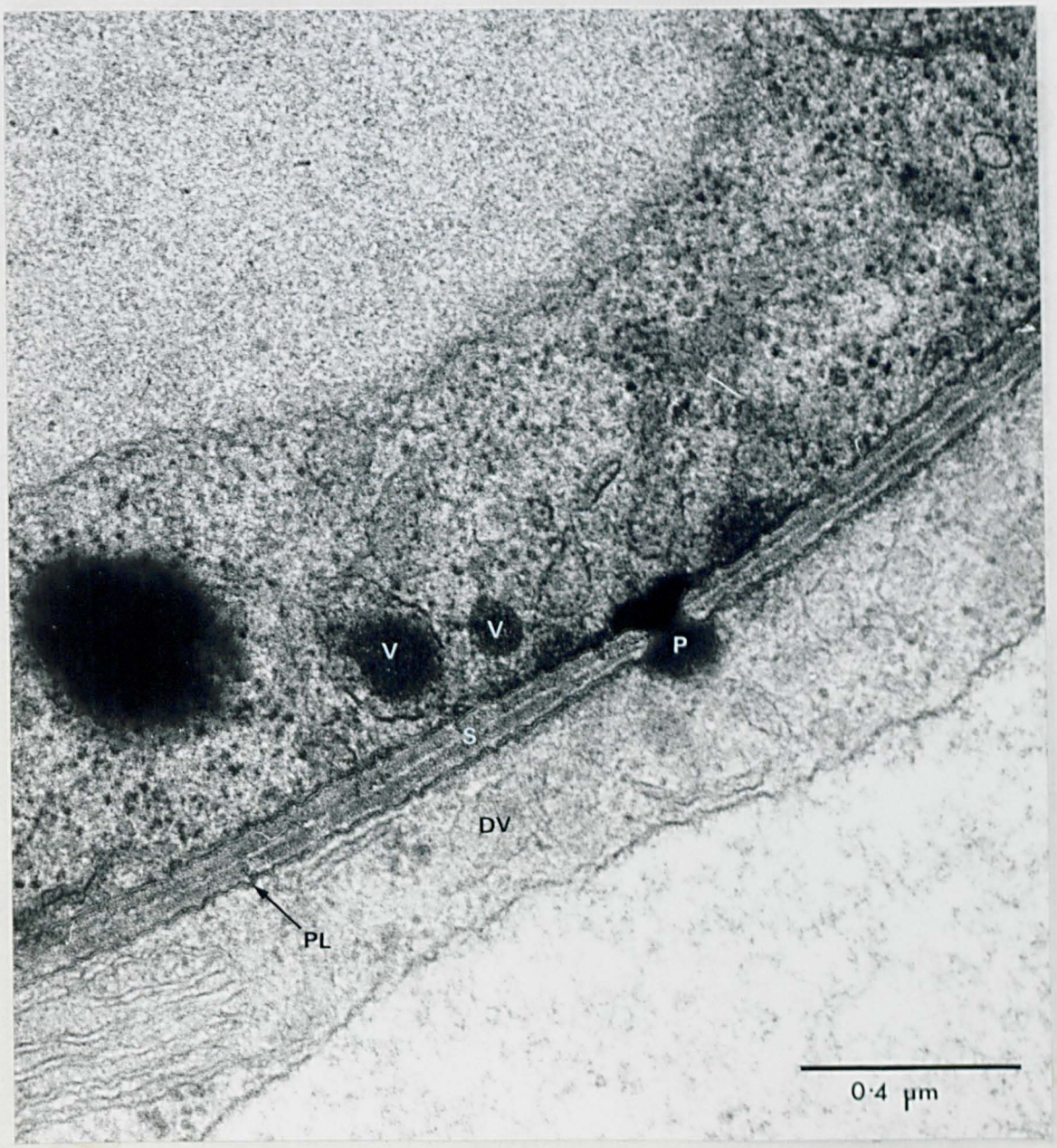
Pl. 8.11 Two electron dense vesicles in close proximity to the septal pore. An electron dense substance, associated with an empty vesicle with a ruptured wall, occupies the septal pore. Vesicles lie in the peripheral position around a cytoplasmic matrix (contains no glycogen granules) on one side of the septum. X 110617.

Key. EDS, electron dense substance; EV, empty vesicle with a ruptured wall; GG, glycogen granules; M, cytoplasmic matrix; V, vesicles.



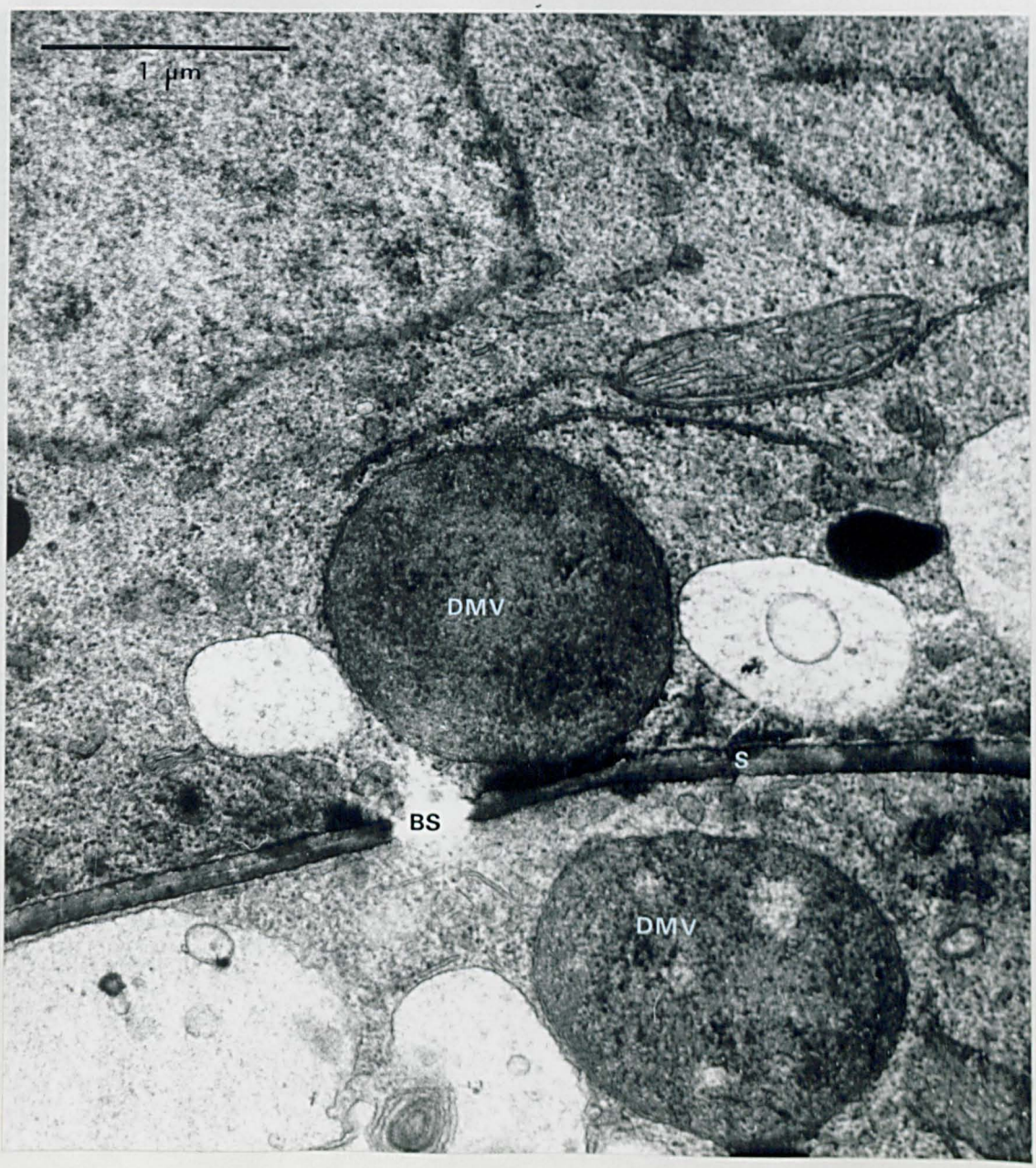
Pl. 8.12 An electron dense substance (believed to be a vesicle) occupies the septal pore. The vague outline of electron dense bands are visible around the pore. Vesicles lie near the pore, one with a well defined internal patterning. X 188873.

Key. B, electron dense band; CL, crystalline lattice; EDS, electron dense substance (vesicle); S, septum; V, vesicles.



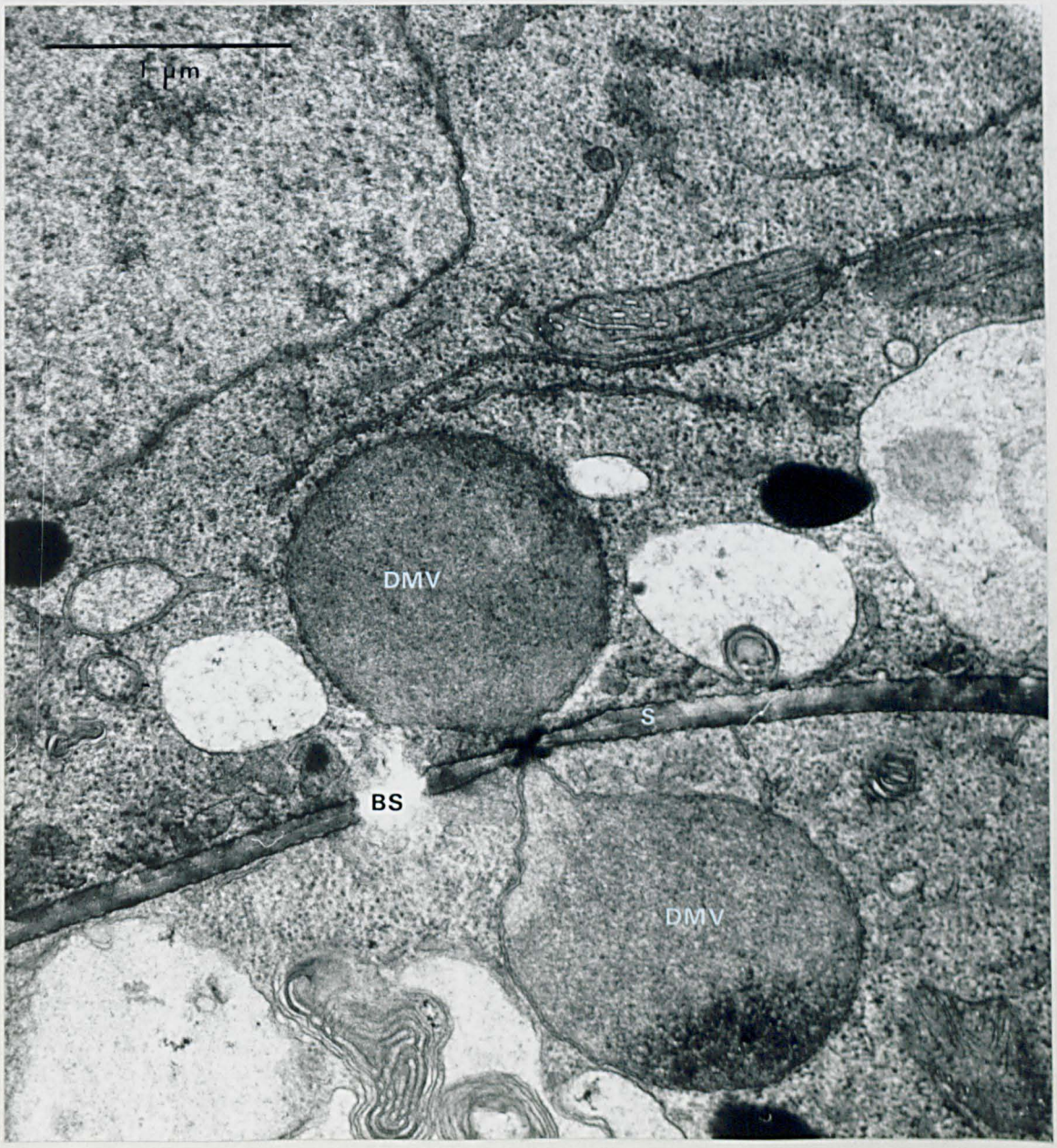
Pl. 8.13 The septal pore is occupied by an electron dense pore plug. Vesicles, some degenerating, lie near the pore area. X 83750.

Key. DV, degenerating vesicles; P, pore plug; PL, plasmalemma; S, septum; V, vesicles.



Pl. 8.14 Two large double membraned vesicles lie close to the septal pore area (indicated by a narrowing of the septum). X 37158.

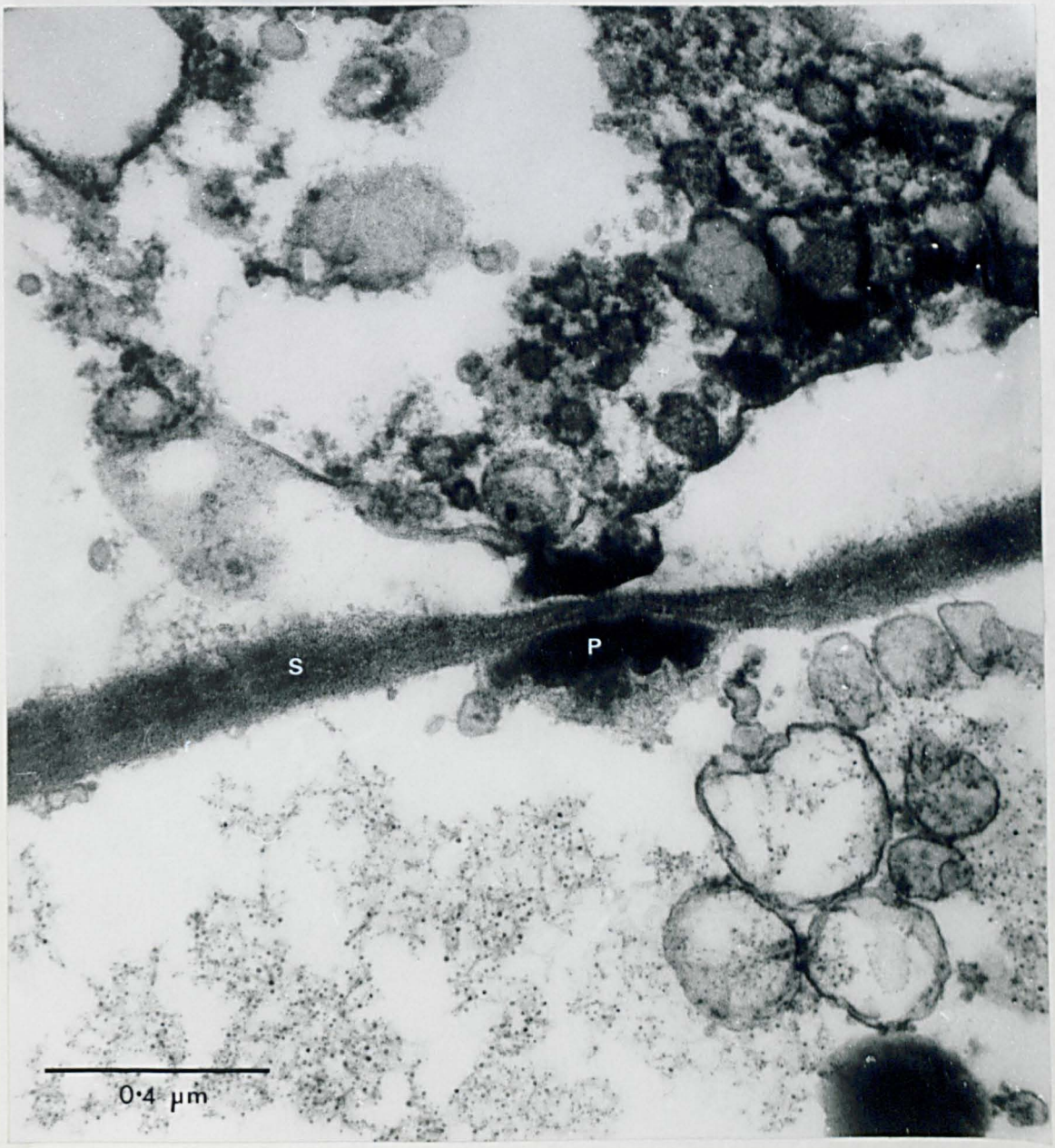
Key. BS, break in septum (believed to have occurred as a result of mechanical damage); DMV, double membraned vesicles; S, septum.



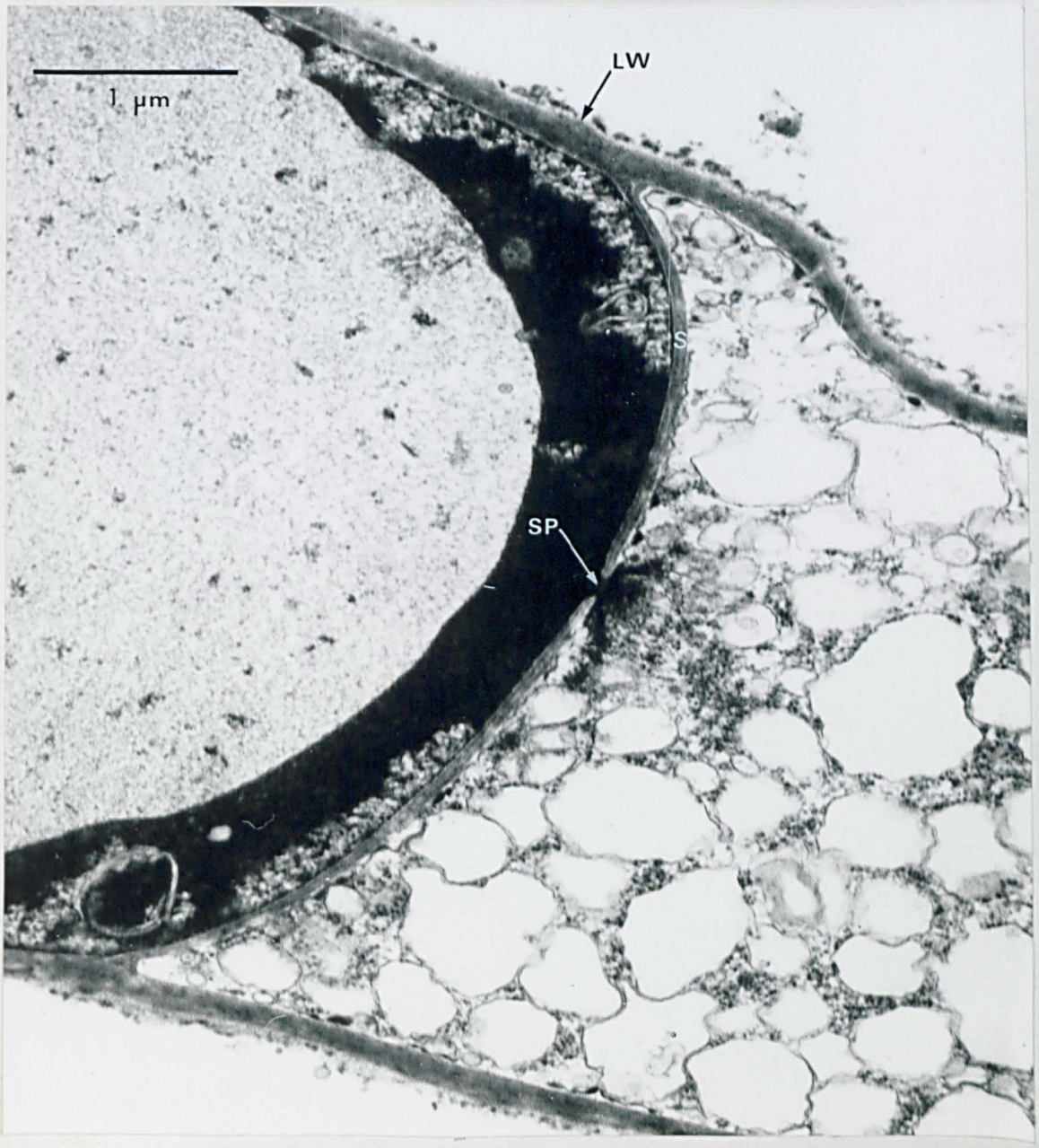
Pl. 8.15 One large double membraned vesicle apparently passing through an indented, electron dense septal pore plug. X 37158.

Key. BS, break in septum (believed to have occurred as a result of mechanical damage); DMV, double membraned vesicle; S, septum.

Pl. 8.16 A large electron dense plug lies in the septal pore between two degenerating hyphal cells. The plasmalemma is separating from the septum. X 22337.
Key. P, pore plug; PL, plasmalemma; S, septum.



Pl. 8.17 A large electron dense plug in the septal pore area (indicated by a narrowing of the septum) between two degenerating hyphal cells. X 83750.
Key. P, pore plug; S, septum.



Pl. 8.18 A septal pore (without a plug) between two degenerating hyphal cells. X 31443.

Key. LW, longitudinal wall; S, septum; SP, septal pore.

SUMMARY.

1) The disease symptoms of the two rust fungi Uromyces dianthi and Puccinia arenariae were described, and host range experiments performed. The epidemiology of P. arenariae was also studied.

2) The morphology of the urediospores and teliospores of U. dianthi and teliospores and basidiospores of P. arenariae, resembled the description given by other workers. The scanning electron microscope showed that no external morphological feature indicated the location of germ pores on U. dianthi urediospores, and that no splitting or distortion of the spore wall occurred adjacent to emerging germ-tubes. Unusually ridge encircled, double spine ornamentations were observed.

3) Experiments revealed that the optimum germination temperature was approximately 20°C for P. arenariae teliospores and 20-22.5°C for U. dianthi urediospores. The latter were found to germinate best in buffer solutions of pH 5.2, and their exudates to contain a self germination inhibitor. This was shown to be thermostable and partially volatile. When distilled, most inhibitory action was detected in the last fraction to come over.

4) Saprophytic growths of U. dianthi were initiated and grown on artificial media from aseptic urediospores. Extended periods of incubation were necessary for the development of the colonies. Media containing yeast extract, peptone and casein hydrolysate, singly and in combination, initiated and supported the cultures. The initiation procedure, the saprophytic hyphae, and spore-like cells found in maturing colonies were described. Attempts to initiate axenic cultures of P. arenariae from basidiospores failed.

5) The optimum growth of U. dianthi in vitro was found to be a temperature of 18°C on media of initial pH 6.0 containing nitrogen culture nutrients at a concentration of 10 g/1. Evans' Peptone was shown to be the best commercial nitrogen medium tested for culturing U. dianthi and methionine the only amino acid (of the ones tested) to support growth. Saprophytic growth was possible on media containing upto and including 200 g of sucrose per litre of medium. Glucose, fructose, maltose, mannose and mannitol could replace sucrose in the medium as the carbohydrate source.

6) The cessation of growth of U. dianthi colonies was related to hyphal differentiation and the production of a growth inhibitor. The cause of growth cessation was believed to be due to a build up of the hydrogen ion concentration in the medium to a level toxic to saprophytic growth. The orange pigment was thought to have an insignificant role in the growth cessation process.

7) Saprophytic growths of U. dianthi were found to be useful in determining the relative in vitro effects of systemic fungicides against rusts.

8) Electron microscope studies of the septal pore areas in saprophytic U. dianthi hyphae revealed that initially a cytoplasmic matrix plug prevented migration of organelles between cells, and this was later replaced by an electron dense plug. The effectiveness of the latter type of plug was questioned. A possible plugging process was described and the phylogenetic importance of the work discussed.

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